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UNIVERSITATIS HELSINKIENSIS

Kuolema kuittaa univelat?

EFFECTS OF CUMULATIVE SLEEP LOSS ON IMMUNE FUNCTIONS AND LIPID METABOLISM

Vilma Aho

**Department of Physiology, Medicum, Faculty of Medicine,
University of Helsinki, Helsinki, Finland**

**Doctoral Program Brain&Mind, Doctoral School in Health Sciences,
University of Helsinki, Helsinki, Finland**

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Supervisors:
Adjunct Professor (Docent) Tarja Stenberg (Porkka-Heiskanen), M.D., Ph.D.
Henna-Kaisa Wigren, Ph.D.
Department of Physiology, Faculty of Medicine, University of Helsinki

Thesis committee:
Professor Esa Korpi, M.D., Ph.D.
Department of Pharmacology, Faculty of Medicine, University of Helsinki

Professor Pertti Panula, M.D., Ph.D.
Department of Anatomy, Faculty of Medicine, Neuroscience Center, University of Helsinki

Reviewed by:
Docent Maarit Hölttä-Vuori, Ph.D.
Department of Anatomy, Faculty of Medicine, University of Helsinki
Minerva Foundation Institute for Medical Research, Helsinki

Professor Paula Salo, Ph.D.
Finnish Institute of Occupational Health, Helsinki and Turku
Department of Psychology, University of Turku, Turku, Finland.

Opponent:
Professor Kenneth P. Wright Jr., Ph.D.
Department of Integrative Physiology, University of Colorado, Boulder, CO, USA

Custos:
Professor Antti Pertovaara, M.D., Ph.D.
Department of Physiology, Faculty of Medicine, University of Helsinki

Cover illustration:

Cordial Sleep (Gene expression in sleep restriction vs baseline as a heartfelt volcano plot) by Vilma Aho (2016)

To my parents & grandparents

"Kuolema kuittaa univelat"

Finnish proverb, origin unknown

Literal translation: *"Death settles sleep debts"*

Translation: *"Time enough to rest when dead" (or at least thesis submitted)*

"Περὶ δὲ ὕπνου καὶ ἐγρηγόρσεως ἐπισκεπτέον τίνα τε τυγχάνει ὄντα, καὶ πότερον ἴδια τῆς ψυχῆς ἢ τοῦ σώματος ἢ κοινά, καὶ εἰ κοινά, τίνος μορίου τῆς ψυχῆς ἢ τοῦ σώματος, καὶ διὰ τίν' αἰτίαν ὑπάρχει τοῖς ζῴοις· καὶ πότερον ἅπαντα κεκοινώνηκεν ἀμφοτέρων, ἢ τὰ μὲν θατέρου τὰ δὲ θατέρου μόνον, ἢ τὰ μὲν οὐδετέρου τὰ δὲ ἀμφοτέρων·"

Ἀριστοτέλης, *Περὶ ὕπνου καὶ ἐγρηγόρσεως*

"With regard to sleep and waking, we must consider what they are: whether they are peculiar to soul or to body, or common to both; and if common, to what part of soul or body they appertain: further, from what cause it arises that they are attributes of animals, and whether all animals share in them both, or some partake of the one only, others of the other only, or some partake of neither and some of both."

Aristotle, *On Sleep and Wakefulness*, 350 BCE (English translation by J. I. Beare)

"Saaliseläimet nukkuvat vähemmän kuin pedot, lehmä nukkuu vähemmän kuin ihminen ja mies vähemmän kuin nainen."

Merikanto I, Partonen T, Lahti T. Evoluution säilyttämä uni (Evolution of sleep). Duodecim 2011, 127: 57-64.

Table of Contents

List of Original Publications

I

Partial Sleep Restriction Activates Immune Response-Related Gene Expression Pathways: Experimental and Epidemiological Studies in Humans. Aho V*, Ollila HM*, Rantanen V, Kronholm E, Surakka I, van Leeuwen WM, Lehto M, Matikainen S, Ripatti S, Harma M, Sallinen M, Salomaa V, Jauhiainen M, Alenius H, Paunio T, Porkka-Heiskanen T. PLoS One 2013, 8: e77184.

II

Prolonged sleep restriction induces changes in pathways involved in cholesterol metabolism and inflammatory responses. Aho V*, Ollila HM*, Kronholm E, Bondia-Pons I, Soininen P, Kangas AJ, Hilvo M, Seppala I, Kettunen J, Oikonen M, Raitoharju E, Hyotylainen T, Kahonen M, Viikari JS, Harma M, Sallinen M, Olkkonen VM, Alenius H, Jauhiainen M, Paunio T, Lehtimaki T, Salomaa V, Oresic M, Raitakari OT, Ala-Korpela M, Porkka-Heiskanen T. Sci Rep 2016, 6: 24828.

III

Homeostatic response to sleep/rest deprivation by constant water flow in larval zebrafish both in the dark and light conditions. Aho V, Vainikka M, Puttonen HAJ, Ikonen HMK, Panula P, Porkka-Heiskanen T, Wigren H-K. *Submitted Manuscript.*

*These authors share equal contribution in the publication.

None of the publications have been used in other dissertations.

These studies are referred in the text by their roman numerals (Study I, II, III).

Abstract

Sleep is an essential physiological function. It is conserved across the animal kingdom; no animal species studied has been shown not to sleep. The timing of sleep and wakefulness is regulated by two processes. The circadian process works like a clock and accounts for the timing of sleep and arousal, synchronised by the light–dark rhythm. The homeostatic process acts like an hourglass balancing the amount of sleep vs wakefulness. In case waking is prolonged, the homeostatic sleep need drives more sleep to take place, i.e. the sleep rebound. The homeostatic aspect of sleep can be studied by restricting sleep in laboratory conditions and assessing the effects on e.g. cognitive functions and physiological processes.

Sleep is not merely a function of the brain, occurring in the brain and for the brain. The brain acts in concert with other organs and tissues in physiological and pathophysiological processes. During the recent decades, epidemiological studies have suggested that there is a connection between short or insufficient sleep with higher mortality. Increased risk for cardiovascular diseases, atherosclerosis, type II diabetes, and obesity has been reported in individuals who sleep less than the average. Laboratory studies have partly supported these findings, suggesting a causative role of sleep loss in the development of metabolic diseases, particularly type II diabetes. Experimental sleep restriction has been shown to alter glucose metabolism towards insulin resistance. Studies on the effects of sleep loss on lipid metabolism have been more inconclusive. Sleep is tightly interconnected with the immune system. Experimental sleep restriction increases proinflammatory cytokines, which in turn promote sleep.

Atherosclerosis is the pathophysiological process underlying ischaemic heat disease and stroke, the two leading causes of death worldwide. The immune system plays a major role in the development of this metabolic disease characterised by plaque-formation in the arterial walls. Altered cholesterol transport by low density and high density lipoproteins (LDL and HDL) triggers an immune response involving macrophages and other white blood cells. Chronic low-grade inflammation has been shown to in turn predict future cardiovascular diseases. Thus, the development of atherosclerosis is a complex process with both metabolic and immunological components.

In the current thesis, I have investigated the effects of sleep loss on gene expression and metabolites in the blood, focusing on changes that may participate in the development of cardiovascular diseases, especially atherosclerosis. Short-term sleep loss was studied in carefully controlled laboratory conditions with an experimental protocol simulating a working week with restricted sleep (4 h sleep/night for 5 nights; N=21). Sleep loss occurring chronically in real-life conditions was assessed in two Finnish epidemiological cohorts. Subjective sleep insufficiency (SSI) was estimated using questionnaire information on sleep, and a prevalence of 16-18% was found in these samples (FINRISK2007/DILGOM; N=518; SSI 16%, and Young Finns Study, YFS; N=2221;

SSI 18%). In both the experimental and epidemiological samples, whole-genome expression profiles were assessed with RNA microarrays and serum lipoprotein profiles with NMR metabolomics.

Immune response-related gene pathways were enriched among transcripts with higher expression in experimental sleep loss. Pathways involved in reverse cholesterol transport (RCT) were down-regulated in both experimental and epidemiological sleep loss. Concentration of large high density lipoprotein (HDL) particles was lower in subjects with SSI, even though in experimental sleep loss the low density lipoproteins (LDL) decreased. Up-regulation of low-grade inflammation-related pathways, and down-regulation of RCT-related pathways with decreased serum large HDL in chronic sleep loss may participate in the development of cardiovascular diseases, such as atherosclerosis.

Experimental and epidemiological sleep studies in human volunteers can complement each other, but still yield information mostly from blood samples. Other methods are needed to elucidate mechanisms involving e.g. the liver. As sleep is a complex phenomenon involving synchronised activity of neuronal networks and integration with other systems and organs, it is not feasible to be studied *in vitro*, i.e. in cultured cells. Thus, animal models are needed to further study the effects of sleep loss at the level of molecular mechanisms.

Zebrafish is a small diurnal vertebrate whose genome has been sequenced. It has a short generation time, readily available genetic tools, and it is well suited for *in vivo* imaging studies thanks to its transparent larval stage. Sleep – or sleep-like states – have been reported in this species using behavioural criteria. According to these studies, adult and larval zebrafish exhibit behavioural quiescence periods with circadian timing and increased arousal threshold. However, the homeostatic sleep rebound after prolonged wakefulness has not been unquestionably proven in this species. To confirm sleep homeostasis and validate this model for further studies on the effects of sleep loss, I developed a method for naturally prolonging the waking activity of zebrafish larvae. After 6 hours of this water flow protocol applied during the night, the larvae showed less responses to sensory stimuli than control larvae. Thus, I suggest that zebrafish larvae do have sleep homeostasis and they can be a useful model to study the sleep loss-related mechanisms involved in disease development.

Tiivistelmä (Abstract in Finnish)

Aristoteles aprikoi jo antiikin Kreikassa 300-luvulla eaa., onko uni mielen, ruumiin vai kenties molempien toiminto. Aivoihin keskittyvät teoriat ovat sittemmin olleet unitutkimuksessa pitkälti vallalla. Viime vuosikymmeninä kiinnostus unen ja kehon toimintojen yhteyttä kohtaan on kuitenkin herännyt uudelleen. Immuunijärjestelmän on havaittu olevan tiiviissä vuoropuhelussa unen säätelyn kanssa. Tulehdusta välittävät tekijät (proinflammatoriset sytokiinit) lisääntyvät univajeessa. Toisaalta nämä samat viestimolekyylit myös lisäävät unta, kuten arkielämässä saattaa bakteeri- tai virusinfektioiden yhteydessä havaita. Vaikka univajetilassa ei ole infektiota, se näyttää aiheuttavan elimistössä puolustusreaktion. Alkuperäinen immuunijärjestelmän aktivaation laukaiseva tekijä ei ole kuitenkaan tiedossa.

Väestötutkimuksissa on havaittu yhteys lyhyen tai riittämättömän unen ja kohonneeseen kuolleisuuden välillä. Myös sydän- ja verisuonitautiriski on joidenkin tutkimusten mukaan keskimääräistä korkeampi vähän nukkuvilla. Näiden epidemiologisten löydösten selittäjiksi on ehdotettu monia tekijöitä univajeen yhteydessä usein esiintyvistä epäterveellisistä elintavoista fysiologisiin tekijöihin. Kokeelliset tutkimukset ovat osin tukeneet väestötason havaintoja. Univajeen on osoitettu mm. nostavan verenpainetta ja ajavan hiilihydraattiaineenvaihduntaa insuliiniresistenssin suuntaan lisäten kakkostyypin diabeteksen riskiä. Rasva-aineenvaihduntaa on tutkittu vähemmän, ja tulokset ovat olleet ristiriitaisia.

Tämän väitöskirjatutkimuksen osatöissä selvitin univajeen aiheuttamia muutoksia ihmisen immuunijärjestelmässä ja aineenvaihdunnassa. Tutkin univajeen vaikutuksia sekä tarkoin kontrolloiduissa kokeellisissa olosuhteissa (laboratoriossa simuloitu vähäuninen työviikko, unta 4 h/yö viiden yön ajan, N=21) että väestötasolla (kansallisen FINRISKI 2007 -terveystutkimuksen osaotos, N=472, sekä Lasten Sepelvaltimotaudin Riskitekijät -aineiston 2007-aikapiste, N=2221). Keskityin erityisesti muutoksiin, jotka saattavat osallistua sydän- ja verisuonitautien kehitykseen.

Tulokset osoittivat, että unen rajoittaminen kokeellisesti terveillä koehenkilöillä aktivoi immuunijärjestelmän geenien ilmentymisen tasolla. Kolesterolin kuljetukseen osallistuvat geenit olivat vähemmän aktiivisia univajeisilla sekä kokeellisessa univajeessa että väestöaineistoissa. Veren lipoproteiinitasoissa kokeellinen univaje laski LDL-partikkeleiden määrää, kun taas väestötasolla suuria HDL-partikkeleita oli vähemmän riittämättömästi nukkuvilla. Ehdotan, että immuunijärjestelmän aktivoituminen univajeessa muuttaa aineenvaihdunnan säätelyä. Tämä näkyy lyhyellä aikavälillä LDL:ien vähentymisenä, mikä perinteisesti tulkitaan sydän- ja verisuonitautiriskiä vähentäväksi. Univajeen ja tulehdustilan kroonituessa kolesteroliaineenvaihdunta saattaa kuitenkin kääntyä epäedulliseen suuntaan ja altistaa sydän- ja verisuonitautien kehittymiselle yhdessä muiden riskitekijöiden kanssa.

Kokeelliset ja väestötason tutkimukset täydentävät toisiaan tarjoten tietoa sekä valvotuista laboratorio-olosuhteista että tosielämän pitkäaikaisvaikutuksista. Ihmisillä tehtävissä tutkimuksissa saadaan yleensä kuitenkin tietoa lähinnä verinäytteistä. Vaikka veressä kiertävät valkosolut ovatkin monien sairauksien keskiössä, myös muut elimet, kuten maksa, ovat olennaisia näiden monimutkaisten prosessien säätelyssä. Koska uni liittyy hermoverkkojen yhteistoimintaan – ja laajemmin aivojen ja muiden elimien yhteistoimintaan – ei sen tutkiminen *in vitro* eli soluviljelmissä petrimaljoilla ole mielekästä. Täten eläinmallit ovat tarpeen, kun selvitetään univajeen ja tautiriskin yhteyden taustalla vaikuttavia molekyylitason mekanismeja.

Aristoteles pohti, nukkuvatko kaikki eläimet ja onko eri lajien nukkuminen samankaltaista ja lähtöisin samasta tarpeesta. Hän esitti kalojen käyttäytymisen havainnointiin perustuen, että kalojen voidaan todeta nukkuvan. Havainnot perustuivat kalojen ajoittaiseen paikallaanoloon, johon liittyi myös aistien osittainen sulkeminen. Tähän yhdistyi myös lajityypillisiä nukkumisasentoja ja -paikkoja. 1980-luvulla esitetyt käyttäytymiskriteerit unen määrittämiseen ilman aivosähkökäyrää (EEG) perustuvat samantyyppiseen havainnointiin. Näillä kriteereillä on raportoitu nukkumista tai nukkumisenkaltaisia tiloja monenlaisilta eläinlajeilta, mukaan lukien kaloilta, banaanikärpäsiltä ja sukkulamadoilta.

Seeprakala on biolääketieteellisessä tutkimuksessa verrattain paljon käytetty mallieläin, jonka genomi on sekvensoitu. Lajin etuina tutkimuksessa ovat mm. lyhyt sukupolvien väli, poikasvaiheen läpinäkyvyys sekä geneettinen muokattavuus. Tämän päiväaktiivisen selkärankaisen on myös käyttäytymiskriteerien perusteella ehdotettu nukkuvan. Seeprakalalla on valveen ja unen ajoituksesta vastaava sirkadiaaninen järjestelmä eli vuorokausirytmi. Unen säätelyn toinen kulmakivi, homeostaattinen unipaine, vastaa unen ja valveen määrän tasapainosta. Valveen pitkittyessä unipaine kasvaa, minkä tuloksena voidaan havaita enemmän ja/tai syvempää unta. Tätä kutsutaan korvausuneksi, ja se voidaan havaita esimerkiksi kohonneena kynnyksenä reagoida aistiärsykkeisiin. Tätä ns. homeostaattista sleep reboundia ei ollut kiistattomasti todistettu seeprakalalta.

Tässä työssä kehitin seeprakalan poikasille luonnollisen menetelmän valveen pitkittämiseen ja reaktioiden mittaamiseen. Menetelmän avulla sain osoitettua, että kalanpoikaset, joiden unta oli rajoitettu yön aikana, reagoivat vähemmän kuin verrokit. Tämän tulkitsen merkiksi homeostaattisesta sleep reboundista. Seeprakalan poikaset soveltuvat tämän jälkeen ihmisainaistoissa saamieni tulosten tarkempiin mekanismitason tutkimuksiin. Mm. kolesterolin kulkeutumista voi seurata *in vivo* eli elävässä eläimessä ja saada tarkempaa tietoa univajeen aiheuttamista kolesteroliaineenvaihdunnan muutoksista.

Kroonistuessaan univaje saattaa ylläpitää elimistössä matala-asteista tulehdustilaa ja muuttaa kolesteroliaineenvaihdunnan säätelyä, ja siten osallistua sydän- ja verisuonitautien kehittymiseen. Seeprakalan poikaset voivat tarjota mahdollisuuksia taustalla vaikuttavien molekyylitason mekanismien tarkempiin jatkotutkimuksiin.

Abbreviations

ABCA1, ABCG1	ATP-binding cassette transporters A1, G1
apoA-I	apolipoprotein A-I
apoB	apolipoprotein B
APR	acute phase response
BL	baseline
BMI	body mass index (weight/height^2)
CASP1	caspase 1
CD36	cluster of differentiation 36; also known as fatty acid translocase, FAT
CETP	cholesteryl ester transfer protein
CHA	cyclohexyladenosine
CoA	coenzyme A
CRP	C-reactive protein
CTRL	control (group)
DILGOM	Dietary, Lifestyle, and Genetic determinants of Obesity and Metabolic syndrome
dpf	days post fertilisation
EEG	electroencephalography
EXP	experimental (group)
HDL	high density lipoprotein
HMG-CoA	3-hydroxy-3-methyl-glutaryl-coenzyme A
IDL	intermediate density lipoprotein
IFN-γ	interferon gamma
IL	interleukin
IL-1β	interleukin 1 beta
IL1B	gene coding for IL-1β
LCAT	lecithin/cholesterol acyltransferase
LDL	low density lipoprotein
LLC	long latency C-start
LXR	liver X receptor
MYD88	myeloid differentiation primary response gene 88
NF-κB	nuclear factor kappa B
NMR	nuclear magnetic resonance
NPC1	Niemann-Pick disease C1
NPC1L1	Niemann-Pick disease, type C1, gene-like 1
NREM	non-REM (sleep)
PLTP	phospholipid transfer protein
PON1	paraoxonase 1
qPCR	quantitative polymerase chain reaction
RCT	reverse cholesterol transport
RD	rest deprivation
REM	rapid eye movement (sleep)
SLC	short latency C-start
SR	sleep restriction
TLR	toll-like receptor
TNF-α	tumour necrosis factor alpha
TNF	gene coding for TNF-α
VLDL	very low density lipoprotein
YFS	Cardiovascular Risk in Young Finns Study
ω-6 (FA)	omega-6 (fatty acid)

1 Introduction

Although already in 350 BCE Aristotle contemplated whether sleep and waking were "peculiar to soul or to body, or common to both", sleep has long been designated as a feature of the brain, occurring *in* the brain and *for* the brain. Indeed, sleep is required for the various neuronal functions such as learning and memory. And the mystery of switching consciousness reversibly off – and back on again – makes sleep an intriguing tool provided by the nature for studying biological aspects of consciousness. (Hobson 2005.)

However, the brain works not alone but in concert with peripheral systems and organs. During the past few decades, knowledge of the connections between sleep and various peripheral systems has been emerging. Sleep – or lack of sleep – has been reported to have an impact on e.g. immune functions, hormonal regulation, and carbohydrate metabolism (Mullington et al. 2010, Van Someren et al. 2015).

Thinking of evolution, this is hardly a surprise. Timing of physiological functions (such as cell division cycle, copying of genetic material, and gaining/using energy) was important already in unicellular archaea and bacteria without any kind of nervous systems. As more complex organisms have developed, the need for synchronisation of the functions in various different organs has become even more crucial. (Bass & Takahashi 2010, Merikanto et al. 2011.) In most animal species, the timing is orchestrated by two processes, the circadian rhythm and the homeostatic regulation of sleep/wake state. The word *homeostasis* (derived from Greek words ὅμοιος, *homoios*, "similar" and στάσις, *stasis*, "standing still", yielding the idea of "staying the same") stands for mechanisms trying to maintain the system in balance. Living organisms try to keep certain internal conditions (such as temperature, energy, and acidity) relatively stable even in changing environments. The homeostatic process in the two process model of vigilance state regulation works to maintain a balance between sleep and wake. The homeostatic sleep pressure, or sleep need, increases while awake, and decreases towards the baseline level during sleep. (Borbély 1982.)

Sleep is a physiological function found in all animal species studied. But science still hasn't found the ultimate function for it – *why* do we all have to sleep? Theories ranging from energy conservation to synaptic plasticity and memory consolidation have been proposed. As knowledge emerges it looks as there might not be one function, but several. And whether there ever was one primary reason sleep evolved for or not, it is now quite clear that during the course of evolution other functions have been clustered to this period of behavioural quiescence. (Merikanto et al. 2011, Van Someren et al. 2015.)

Some of these functions are connected to systems that are also linked to the development of cardiovascular diseases. Epidemiological research has suggested that people who sleep less have higher cardiovascular and all-cause mortality (Gallicchio & Kalesan 2009, Cappuccio et al. 2010, Grandner et al. 2016). Short or insufficient sleep has been

associated to an increased risk for atherosclerosis, type II diabetes, and obesity (Grandner et al. 2016). Atherosclerosis is the pathophysiological process underlying the two top causes of death worldwide, ischaemic heart disease and stroke. Lipid metabolism and transport, especially cholesterol, are in the centre of atherogenic processes. (Badimon & Vilahur 2012.)

Sleep loss has been found to drive carbohydrate metabolism in the direction of insulin resistance, which may at least partially explain the increased risk for type II diabetes (Hanlon & Van Cauter 2011). Studies on the effects of sleep loss on lipid (fat, cholesterol) metabolism have been more scarce.

The main cardiometabolic diseases have also a strong immunological component (Swirski & Nahrendorf 2013). Low-grade inflammatory state has been shown to play a major role in the pathophysiology of atherosclerosis, type II diabetes, and numerous other diseases (Libby et al. 2002, Hummasti & Hotamisligil 2010). It is also well established that there is a bidirectional connection between sleep and the immune system (Imeri & Opp 2009, Krueger et al. 2011). A humoral regulation of sleep was suggested already in the turn of the 20th century, but neural studies of sleep and arousal largely overshadowed humoral theories for some decades. The first sleep-inducing substance, the "Factor S", was extracted in the 1970s and later characterised as an interleukin 1-inducing immune adjuvant (Mullington et al. 2010). Since then, many laboratory experiments have confirmed that proinflammatory cytokines – body's molecular messengers of inflammation – increase in sleep restriction and induce sleep (Krueger et al. 2011). Epidemiological evidence on the significance of sleep loss-induced inflammation in real-life conditions is emerging, but has been rather indefinite thus far (Mullington et al. 2010, Grandner et al. 2016).

As sleep is a complex function of neuronal networks and even other systems in the whole body, it cannot be studied *in vitro* in cultured cells. Experimental and epidemiological studies in humans provide information mainly from blood samples. Thus, animal models are needed to elucidate the effects of sleep and sleep loss on (human) physiology. This is where zebrafish swims into the picture. This small fish is a diurnal vertebrate that has been shown to have sleep-like states (Zhdanova 2011, Chiu & Prober 2013). After confirming sleep homeostasis in this transparent model, it can be used for studies imaging sleep-related processes *in vivo*. Advantages such as short generation time, small size, well-characterized behavioural repertoire, suitability to high-throughput assays, and transparent larval stage make zebrafish an attractive model organism that can complement the knowledge obtained from mammalian studies. By studying different models from relatively distant branches of the animal kingdom – such as humans, rodents, fish, and insects – it is possible to gain information on the conserved functions of sleep and universal effects of sleep loss. As Aristotle pondered, "with regard to sleep and waking, we must consider what they are" and "from what cause it arises that they are attributes of animals, and whether all animals share in them both, or some partake of the one only, others of the other only, or some partake of neither and some of both."

2 A Review of the Literature

Here I give some background on sleep and cardiovascular diseases, and the processes potentially connecting these, focusing on the immune system and cholesterol metabolism. Furthermore, I introduce zebrafish as a model organism for sleep research.

2.1 Sleep as a physiological function

Sleep is a state of behavioural quiescence, but it is much more than a quiet state. Various physiological functions occur in a larger extent during sleep than wakefulness. Also, sleep is not a unitary state, instead, there are several different levels of sleep. (Hobson 2005.)

2.1.1 Definition and regulation of sleep

Sleep is a state of relative behavioural quiescence and unconsciousness. By definition, sleep is reversible by sensory stimuli with a sufficient intensity. During sleep, arousal threshold is increased, leading to decreased sensory input to cortex, mediated by the thalamus. (Coenen & Drinkenburg 2002.) Sleep and arousal are regulated by reciprocally inhibited neurotransmitter systems, creating a "flip-flop switch" (Saper et al. 2005). Vigilance states can be characterised and detected using measurements of brain activity and/or behavioural criteria.

According to the two process model proposed by Borbély in the 1980s, the timing of sleep and wake is regulated by the circadian and homeostatic processes (Borbély 1982). The circadian rhythm synchronises bodily functions entrained by the light rhythm. The homeostatic sleep pressure accumulates during wakefulness and decreases back towards baseline during sleep. In case wakefulness is prolonged, sleep pressure increases further, promoting deeper and/or longer sleep. This recovery sleep is called the homeostatic sleep rebound. (Borbély et al. 2016.)

2.1.2 Sleep stages

Sleep can be detected by measuring neural oscillations ("brain waves") using electroencephalography (EEG) (Rechtschaffen & Kales 1968, Hobson 2005). During wakefulness, the brain activity manifests as high frequency and low amplitude waves in EEG, while during sleep neurons fire with more synchronisation, and thus lower frequency and higher amplitude waves can be observed in EEG. Rapid eye movement (REM) sleep, or so-called paradoxical sleep, resembles wake in the EEG. The rapid eye movements can be detected by electrooculography (EOG), and most dreams occur during this phase. Non-REM (NREM) sleep in human is divided into three stages based primarily on the exhibition of slow waves. Typically, the sleep stages oscillate throughout the night in cycles of approximately 1.5-2 hours, starting from light NREM sleep stages 1 and 2 and deepening to stage 3 NREM, the slow wave sleep, and ending with a REM sleep episode. The deepest NREM sleep stage normally occurs more in the first sleep cycles when the sleep pressure is high, and decreases towards the end of the night.

Correspondingly, REM sleep and light NREM sleep increase closer to the morning. (Hobson 2005.)

2.1.3 Changes in physiology during sleep

Sleep is connected to other physiological changes in the brain and the whole body. In NREM sleep, the autonomic nervous system balance shifts from sympathetic to parasympathetic dominance. Blood pressure, breathing rate, body temperature, and cortical blood flow decrease. (Parmeggiani 2005.) REM sleep atonia, almost complete paralysis of the body, keeps muscle tone low throughout the body during REM sleep (Siegel 2011). Also the autonomic nervous system regulation is "unplugged" in REM sleep, leading to irregular blood pressure, heart rate, breathing, and body temperature (Siegel 2011). The gold standard of clinical sleep recording, polysomnography (PSG), gathers information via other physiological measurements in addition to the EEG, as its name suggests. These include measurements of electrocardiography (ECG), EOG, muscle movement, breathing, and oxygen saturation. (Carskadon & Rechtschaffen 2011.)

2.1.4 Defining sleep with behavioural criteria

PSG is routinely recorded in humans, many other mammals, as well as some birds and reptiles. In species where EEG-based methods are not available, other criteria for defining sleep and wakefulness are needed. During the past decades, behavioural criteria have been used to characterise sleep or sleep-like states in other animals such as flies, worms, and fish (Campbell & Tobler 1984, Zhdanova 2006, Raizen & Zimmerman 2011).

Some studies rely on measuring movement activity only, and define inactivity bouts of certain duration as sleep. However, these methods, such as the measurements using wearable activity-measuring devices (actigraphs) in humans and locomotor activity tracking by e.g. video recordings in animals, have the limitation that quiet wakefulness can be defined as sleep, and they cannot be applied to animals that move while asleep, such as some birds and marine mammals (Aulsebrook et al. 2016). Actigraphic measurements in humans correlate with polysomnography, but differences occur and sleep stages (especially REM sleep) usually cannot be very reliably distinguished (Ancoli-Israel et al. 2003). In animal studies, measurements of arousal threshold are used to distinguish sleep from inactivity during wakefulness (Hendricks et al. 2000).

2.1.5 Experimental studies of sleep homeostasis

For targeting the homeostatic regulation of sleep, experiments of prolonged wakefulness and measurements of the following sleep rebound are fundamental tools in both humans and animal models. Typically, human subjects can be sleep-deprived for e.g. 24 or 48 hours, or their sleep can be restricted to e.g. 4-6 hours per night for several consecutive days. The former is often called acute or total sleep deprivation. The latter, partial sleep deprivation or sleep restriction, resembles the sleep curtailment that can occur e.g. during busy work schedules in real life.

2.1.6 Self-evaluation of sleep in humans

Besides the objective measurements, sleep is often studied in humans using subjective evaluation. With epidemiological data collections, sleep can be studied in actual real-life conditions. In these studies, sleep is usually neither restricted nor monitored. Instead, self-reported questionnaire information is obtained from the participants. Questions can address e.g. sleep duration, sleep need, sufficiency of sleep, feeling of tiredness etc. (Grandner et al. 2010.). By addressing subjective sleep insufficiency instead of sleep duration it may be possible to better reflect the experimental sleep restriction, where sleep opportunity is shortened from the normal duration of the participants typically leading to insufficient sleep. However, the interpretation and use of different questions assessing sleep duration and sufficiency is still under debate, including their validity in e.g. distinguishing natural short sleepers (individuals who have shorter sleep need) and relevance in detecting associated health risks (Grandner et al. 2010, Irwin et al. 2016).

2.1.7 Epidemiological and experimental study arrangements

Epidemiological studies can be cross-sectional, where the measures of interest (e.g. cholesterol) are compared between individuals grouped by a certain factor (e.g. sufficiency of sleep) at one time point, or prospective, where the effect of a factor (e.g. sufficiency of sleep) can be estimated longitudinally by comparing the change of the measures of interest (e.g. cholesterol) between consecutive time points. Prospective studies may yield predictive estimates and suggest causalities, while cross-sectional studies typically give only information on associations.

In experimental studies, sleep can be modified and monitored. The effect of experimentally-induced sleep loss can be studied longitudinally within-subject taking samples e.g. before and after sleep restriction, and/or between-subjects comparing groups with and without an opportunity to sufficient sleep.

In laboratory studies, it is also possible to carefully control other conditions, such as diet, physical activity, temperature etc., whereas in epidemiological studies various other factors can interfere with the analysis of the effect of sleep loss. The advantages of epidemiological studies include the real-life aspect in addition to an often larger number of subjects, ranging from hundreds to thousands or sometimes even hundreds of thousands, whereas laboratory studies on sleep restriction typically comprise tens of individuals (Irwin et al. 2016).

In this study, the effects of lack of sleep were studied both epidemiologically, using population cohorts with questionnaire information, and experimentally, using prolonged wakefulness in laboratory conditions for humans and zebrafish. The population samples were cross-sectional, comparing individuals reporting insufficient sleep to those reporting sufficient sleep. In the laboratory experiment, we studied the within-subject effect of sleep restriction compared to the baseline of each subject, and the between-subject aspect comparing a sleep-restricted group to a normally-sleeping control group.

The zebrafish experiments compared rest-deprived groups to groups that had had a normal opportunity to sleep.

2.2 Short sleep as a risk factor for cardiovascular diseases

Sleep and circadian rhythms play an important role in managing energy homeostasis in peripheral tissues (Broussard et al. 2012, Depner et al. 2014). Sleep problems, such as insufficient sleep and circadian misalignment, may contribute to metabolic dysregulation (Depner et al. 2014). Sleep loss may cause inflammation, which is also connected to the development to the pathophysiology of various metabolic diseases (Libby et al. 2002, Hummasti & Hotamisligil 2010). Effects of sleep – or sleep loss – on the immune system and glucose metabolism have been established in many studies, while studies on lipid metabolism have been more scarce.

Sleep has been linked to various diseases, including atherosclerosis, cardiovascular diseases, type II diabetes, and obesity (Cappuccio et al. 2008, Knutson & Van Cauter 2008, Cappuccio et al. 2011). These epidemiological findings have been supported and partly explained by experimental sleep restriction studies. Various processes have been suggested to mediate the adverse effects of short sleep on cardiovascular health. These include hypertension (high blood pressure), low-grade inflammation, changes in appetite-controlling hormones, insulin resistance-promoting changes in glucose metabolism, and adverse behavioural choices such as unhealthy diet and sedentary lifestyle (Gangwisch et al. 2006, Mullington et al. 2009, Grandner et al. 2010, Knutson 2013).

Most epidemiological studies have addressed the duration of sleep, comparing individuals with short sleep to those with "normal" or "long" sleep, with varying cut-offs between the groups (Grandner et al. 2010). Only few reports on the association of self-reported insufficient sleep and cardiovascular health have been published (Shankar et al. 2010, Altman et al. 2012).

2.2.1 Sleep, mortality, and cardiovascular diseases

A U-shaped association has been found between self-reported sleep duration and all-cause mortality (Grandner et al. 2010). In several studies, both short sleepers and long sleepers have been reported to have higher mortality than those with a "normal" sleep duration, typically defined as 7-8 hours (Gallicchio & Kalesan 2009, Kronholm et al. 2011). It has been suggested that the risks associated with short and long sleep represent two distinct phenomena with possibly different mechanisms and should be studied separately (Grandner et al. 2010).

Short sleep has been found to be associated to increased risk for cardiovascular diseases and higher cardiovascular mortality in a few studies. In a Finnish population study, the U-shaped association of self-reported sleep duration with all-cause mortality was confirmed in both genders (Kronholm et al. 2011). The highest cardiovascular mortality

risk was found in subjects with the shortest (≤5) and longest (≥10 h) sleep durations. Yet, the independent association of sleep duration with cardiovascular mortality was found significant only in women when pertinent risk factors were included in the hazard model. Also the risk for myocardial infarction has been reported to be higher in short-sleeping middle-aged women than men (Meisinger et al. 2007). Another study found an increased risk for stroke in postmenopausal women with less than 7 h habitual sleep duration (Chen et al. 2008). A recent longitudinal study reported an increased subsequent risk for hypertension and dyslipidaemia after onset of impaired sleep, but not onset of short sleep, after adjusting for other risk factors (Clark et al. 2016). These authors used a cohort of Finnish public sector employees linked to medical registry data, and defined impaired sleep with self-reported insomnia symptoms and dyslipidaemia as need for statin medication.

Risk for coronary artery calcification, a subclinical predictor of coronary heart disease, has been shown to increase with each hour of actigraphy-measured short sleep (King et al. 2008). A study in Japanese population found an association of carotid artery atherosclerosis with longer sleep, but no increased risk with short sleep (Abe et al. 2011). In this study, 6 hours was used as the reference sleep duration, and durations ≤5 h were defined as short sleep and ≥7 h as long sleep.

Thus, the findings regarding cardiovascular mortality and morbidity have not been entirely consistent (Kronholm et al. 2011). Differences in the findings may be due to different populations studied, inconsistent definitions of short sleep, and various cardiovascular endpoints and risk factors measured. Confounding factors, such as age, sex, and other lifestyle factors in addition to sleep may also play a role in the varying findings. Combining adjacent groups with the extreme sleep duration groups may partly mask the mortality risks (Kronholm et al. 2011). Also, the group defined as short sleepers may include so-called 'natural short sleepers' who might have a shorter sleep need (Grandner et al. 2010). Thus, these individuals possibly do not sleep less than they would require.

Only few studies have addressed insufficient sleep instead or in addition to short sleep regarding the association with cardiovascular measures (Grandner et al. 2010). A study in a US national representative sample of over ~370,000 individuals assessed sleep insufficiency by asking how many days the participants felt not having had enough sleep during the past month (Shankar et al. 2010). An association was found for insufficient sleep with self-reported cardiovascular diseases overall, as well as coronary heart disease, stroke, type II diabetes, and obesity. Another questionnaire study in ~30,000 people from the US population using the same question reported that frequent insufficient sleep was associated to self-reported BMI, obesity, type II diabetes, hypertension, hypercholesterolemia, heart attack, and stroke, while sleep insufficiency only to hypertension and hypercholesterolemia (Altman et al. 2012). A study in the Finnish FINRISK 2007 sample also found a negative association between hypertension and self-reported sleep sufficiency using a question on the frequency of sufficient sleep

(Merikanto et al. 2013). These studies suggest that sleep insufficiency and duration are both related to cardiometabolic health outcomes, and demonstrate partly overlapping, partly separate effects (Altman et al. 2012).

2.2.2 Sleep and type II diabetes

Prospective epidemiological studies have reported an increased risk to develop type II diabetes with shorter sleep durations, suggesting a causative connection from sleep loss to type II diabetes (Knutson 2007). An association of self-reported insufficient sleep with type II diabetes has also been reported (Shankar et al. 2010).

Experimental sleep restriction studies have supported the hypothesis of a causative connection, as sleep loss has been shown to alter the regulation of glucose metabolism (Spiegel et al. 1999, Knutson 2007). Partial sleep restriction has been reported to reduce glucose tolerance and insulin response to glucose (Spiegel et al. 1999). The sensitivity to insulin has also been shown to decrease in adipocytes of sleep-restricted subjects (Depner et al. 2014). Decreased insulin sensitivity, i.e. insulin resistance, is the hallmark of the development of diabetes mellitus type II. If the sleep impairment becomes chronic, these metabolic changes may contribute to the development or exacerbation of type II diabetes (Knutson 2007).

2.2.3 Sleep and obesity

A U-shaped association of sleep duration with obesity has also been proposed (Grandner et al. 2012). A meta-analysis in over 600,000 subjects found an increased risk of obesity in adults sleeping less than 5 hours and children sleeping less than 10 hours (Cappuccio et al. 2008). However, another meta-analysis found a consistent association only in children and young adults, but not in older adults (Nielsen et al. 2011). One study found a negative correlation between body mass index (BMI) and sleep duration in men, while a U-shaped association was observed in women (Kripke et al. 2002). An association between subjective sleep insufficiency and obesity has also been suggested (Shankar et al. 2010).

The reports of an association between short sleep and obesity have led to laboratory studies addressing the mechanisms of this connection. Leptin, a "satiety hormone" secreted by the adipocytes ("fat cells"), decreases appetite, while the "hunger factor" ghrelin has the opposite effect. Genetic deficiencies in leptin function have been shown to result in significant obesity in mice and humans. Obesity, in turn, has been suggested to cause a leptin-resistance state (resembling insulin-resistance), creating a vicious cycle of excess dietary intake (Considine 2005).

Sleep deprivation has been reported to decrease leptin and increase ghrelin (Spiegel et al. 2004, Hanlon & Van Cauter 2011). These hormonal changes may result in increased dietary intake and participate in the higher risk for developing obesity. In line with this hypothesis, some studies have reported that sleep-deprived subjects tend to eat more and choose more calorie-dense foods in laboratory conditions (Depner et al. 2014).

Despite a small increase in energy consumption by sleep deprivation compared to sleep, it seems that the energy intake often is overcompensated (Markwald et al. 2013). This may create a positive energy balance, leading to overweight and obesity on the long run.

2.3 Atherosclerosis as a pathophysiological process

Cardiovascular diseases, namely ischaemic heart disease and stroke, are the leading causes of death, currently accounting for over ¼ of deaths worldwide. The underlying cause of myocardial infarction (heart attack) and stroke is the accumulation of lipid-rich plaques in the arterial walls, which may lead to blockage in the blood flow (Badimon & Vilahur 2012). The plaque build-up process, atherosclerosis, is a chronic disease with metabolic and inflammatory origin. It involves circulating lipoproteins and various cell types, such as the endothelial cells and smooth muscle cells of the vascular wall, monocytes differentiating to macrophages and then to foam cells, and several other types of leukocytes with pro or anti-inflammatory/atherogenic functions (Hansson & Libby 2006, Rosenson et al. 2012).

Risk for developing atherosclerosis is strongly associated to lifestyle. In addition to age and male sex, smoking, diabetes-associated obesity, unhealthy diet, and sedentary lifestyle are prominent risk factors (Fruchart et al. 2004). Short sleep has been suggested as another risk factor for atherosclerosis (King et al. 2008). Physiological risk factors and biomarkers include hypertension, dyslipidaemia, and chronic low-grade inflammation (Fruchart et al. 2004). Genetic predisposition also affects the risk of developing atherosclerosis, and the use of genetic testing for atherosclerosis is increasing in clinical diagnostics (Paynter et al. 2016).

Inactivating mutations in genes coding for cholesterol transport-related proteins, such as ATP-binding cassette (ABC) transporters and low density lipoprotein receptor (LDLR), cause deficiencies in lipid metabolism leading to increased risk for atherosclerosis (Fitzgerald et al. 2010, Iatan et al. 2012, Paynter et al. 2016). In addition to these severe hereditary phenotypes, a growing number of genetic polymorphisms mainly connected to the regulation of metabolic and immune response processes have been shown to contribute to the risk burden along with lifestyle factors (Incalcaterra et al. 2013, Musunuru & Kathiresan 2016, Paynter et al. 2016). Thus, atherosclerosis is a complex disease affected by both lifestyle and genetic factors, and its development involves metabolic and inflammatory components.

2.3.1 Cholesterol metabolism and transport

Lipid metabolism is in the centre of the development of atherosclerotic lesions. Dysregulation of lipids, especially cholesterol, leads to build-up of plaques in the vascular wall. Lipoproteins carrying cholesterol and other lipids in the blood are major regulators of lipid and cholesterol homeostasis, and thus pivotally involved in the atherosclerotic processes. Elevated low density lipoprotein (LDL) concentration is a risk factor for atherosclerosis, while higher concentration of high density lipoprotein (HDL) is

associated to lower risk (Gordon et al. 1977, Fruchart et al. 2004, Emerging Risk Factors Collaboration et al. 2009, Badimon & Vilahur 2012).

Cholesterol is commonly known as an "evil villain" of health because of its connections to metabolic diseases, such as atherosclerosis. But cholesterol is an enemy of our health only when accumulating in excess in wrong places. In normal physiology, cholesterol is required by the tissues as an important component of cell membranes and a precursor for vitamin D, bile acids, and natural steroid hormones, such as oestrogens, androgens, and corticosteroids (Yu et al. 2014, Alphonse & Jones 2016). Intestinal absorption, endogenous synthesis, transport, and elimination of cholesterol are complex processes with tight regulation to control the level of cholesterol in tissues. However, in the course of human evolution, before the modern cholesterol-rich diets, the main risk regarding cholesterol has been deficiency, not excess. Thus, our metabolism is often unable to handle the high amounts of cholesterol and lipids obtained from modern diet, leading to accumulation and pathogenic processes of atherosclerosis. (Davalos & Fernandez-Hernando 2013.)

In addition to uptake from diet, cholesterol is also *de novo* synthesised by cells. Cholesterol biosynthesis is controlled by various genetic, dietary, and physiological factors, such as circadian rhythm. The synthesis pathway consists of over 30 reactions catalysed by more than 15 enzymes, with acetyl coenzyme A (acetyl-CoA) and acetoacetyl-CoA as the initial precursors. The principal rate-limiting enzyme, HMG-CoA reductase, converts 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) to mevalonate, and serves as an important target of the cholesterol-lowering statin drugs. (Alphonse & Jones 2016.)

Hydrophobic (*"water-fearing"*) molecules like cholesterol are not water-soluble, and thus have to be packed in lipoprotein complexes, such as LDL and HDL, for transportation in the aqueous blood stream. Cholesterol obtained from the diet is first packed in chylomicrons and very low density lipoproteins (VLDL) and transported to the liver. Low density lipoprotein particles (LDL) carry the cholesterol from the liver to the tissues. Excess cholesterol is transported back to the liver packed in high density lipoproteins (HDL). This HDL-mediated process is called reverse cholesterol transport (RCT) (Figure 1). Liver metabolises the excess cholesterol to bile acids for use in the gut or excretion in faeces.

Cholesterol carried by LDL is often nominated as the "bad guy" in the complex picture of cholesterol transport. Risk for atherosclerosis is associated to high LDL in epidemiological studies (Fruchart et al. 2004). In clinical diagnostics, high concentration of LDL and its main apolipoprotein, apoB, are considered risk factors for cardiovascular diseases (Wilson et al. 1998). Elevated plasma cholesterol promotes entrapment of cholesterol-carrying LDL in the arterial wall where it becomes exposed to oxidation and other modifications, which may contribute to the activation of the inflammatory responses and thus the pathophysiology of atherosclerosis (Hansson & Libby 2006).

Current medications for atherosclerosis, including statins, rely mainly on decreasing LDL cholesterol (Amarenco et al. 2004, Tian et al. 2012).

Cholesterol efflux from cells to HDL is the first step in RCT (Rosenson et al. 2016) (Figure 1). The main apolipoprotein in HDL, apoA-I, is involved in the regulation of cholesterol efflux. Many of the intrinsic anti-inflammatory, anti-oxidative, and anti-bacterial properties of HDL are also accredited to apoA-I, though a complex cluster of proteins is involved in these functions (Rosenson et al. 2016). Higher HDL levels have been shown to associate with lower risk for cardiovascular diseases (Gordon et al. 1977, Emerging Risk Factors Collaboration et al. 2009). On account of the epidemiological findings and the above-mentioned functions, HDL has traditionally been called the "good cholesterol (carrier)".

The biogenesis of HDL requires apoA-I to interact with the ATP-binding cassette transporter A1 (ABCA1), leading to the transfer of cholesterol and phospholipids to lipid-poor apoA-I (Rader & Tall 2012, Zannis et al. 2015). Lecithin/cholesterol acyltransferase (LCAT) esterifies the cholesterol in lipidated apoA-I, creating a spherical HDL particle. Inactivating mutations in the genes coding for apoA-I, ABCA1, or LCAT can prevent the formation of HDL (Zannis et al. 2015).

ATP-binding cassette transporter G1 (ABCG1) promotes efflux of cellular cholesterol to HDL particles, but not lipid-free apoA-I (Rader & Tall 2012, Zannis et al. 2015). Variants of *ABCG1* have been associated to HDL cholesterol level and coronary artery disease (Zannis et al. 2015).

HDL and other lipoproteins undergo modifications e.g. by lipid transporter proteins. Cholesteryl ester transfer protein (CETP) can transfer cholesterol esterified by LCAT from HDL to VLDL or LDL particles in exchange for triglycerides (Rader & Tall 2012, Zannis et al. 2015). Phospholipids can be transferred from VLDL to HDL by phospholipid transfer protein (PLTP) (Zannis et al. 2015). These actions cause changes in the size and functional properties of the particles, and thus participate in the regulation of plasma lipid levels and production of potentially atherogenic or anti-atherogenic lipoproteins (Zannis et al. 2015).

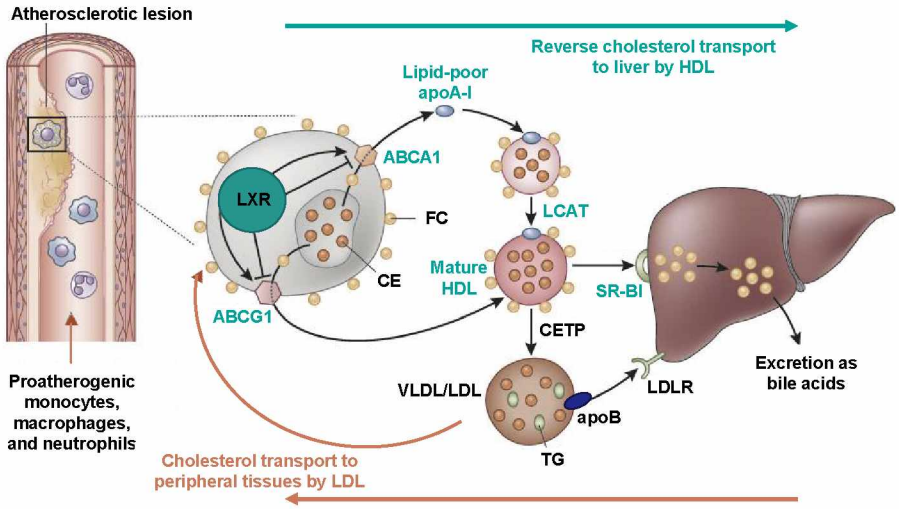

Figure 1. Reverse cholesterol transport (RCT)
A simplified model of the roles of high density lipoproteins (HDL), very low and low density lipoproteins (VLDL/LDL), apolipoproteins apoA-I and apoB, free cholesterol (FC), esterified cholesterol (CE), ABC transporters (ABCA1, ABCG1), lecithin-cholesterol acyltransferase (LCAT), cholesteryl ester transfer protein (CETP), scavenger receptor B1 (SR-BI), low density lipoprotein receptor (LRLR), and liver X receptor (LXR) in RCT. Modified from (Rader & Tall 2012).

However, there is a complex relationship of disease development and HDL particles with different sizes, oxidation levels, and other modifications (Rosenson et al. 2016). HDL-increasing medications have not proved very successful in the treatment of atherosclerosis thus far (Tariq et al. 2014). It is not only its role in the reverse cholesterol transport that makes HDL a player in the anti-atherogenic team, but also its anti-inflammatory and antioxidative properties (Rosenson et al. 2016). Paraoxonase 1 (PON1) has been suggested to be one major actor involved in the antioxidative functions of HDL (Soran et al. 2015). PON1 activity has been shown to be inversely associated to coronary events (Soran et al. 2015). Methods separating different sizes and other properties of lipoproteins may provide new information on the relationship of these particles with disease risk and development (Rosenson et al. 2013, Soininen et al. 2015).

2.3.2 Inflammatory activation

Inflammation is an important process in host defence against pathogens and injury, but it can also contribute to the development of numerous diseases, including atherosclerosis (Hansson & Libby 2006, Swirski & Nahrendorf 2013). A theory of inflammation as the driving force of atherosclerosis was presented already in 1856 by Rudolf Virchow, but the scientific proof started emerging a few decades ago (Poston & Davies 1974). Both innate and adaptive immune responses play a role in the pathophysiology of atherosclerosis (Hansson & Libby 2006, Swirski & Nahrendorf 2013).

LDL modification, especially oxidation, enables cell adhesion molecule expression by endothelial cells and thus accelerated intake of LDL by cells, such as macrophages differentiating from monocytes and migrating into the intima of the vascular wall. In this scavenger receptor and CD36-mediated process, cholesterol accumulates in macrophages, which develop into foam cells, a hallmark of early atherosclerotic lesions (Stary et al. 1994, Hansson & Libby 2006). HDL promotes cholesterol efflux from the macrophage foam cells and transport to the liver (Rosenson et al. 2012). If this process of RCT fails, the foam cells may be destined to apoptosis, driving atherosclerotic lesion-development (Hansson & Libby 2006).

Furthermore, the modified LDL triggers proinflammatory activation via toll-like receptor (TLR) and nuclear factor kappa B (NF-κB)-mediated pathway (Hansson & Libby 2006), leading to the production of proinflammatory chemokines and cytokines, such as interleukin 1 beta (IL-1β) (Stewart et al. 2010).

Acute phase response (APR) is a systemic reaction that plays a role in the host defence. However, APR and inflammation are also involved in the development of many disease states, such as atherosclerosis. C-reactive protein (CRP) is an acute phase protein produced by the liver in response to many acute conditions, such as inflammation, surgical trauma, and myocardial infarction (Koenig 2013). CRP is one of the markers of low-grade inflammation clinically used in cardiovascular risk assessment (Koenig 2013). Inflammatory mediators, such as CRP, IL-1β, and interleukin 6 (IL-6), have been found to predict prospective atherosclerotic complications (Qamar & Rader 2012, Koenig 2013, Zamani et al. 2013). Thus, it has been hypothesised that the causal connection is not only from lipid accumulation to inflammation, but also *vice versa*; inflammation promotes the initiation of plaque-formation (Libby et al. 2002).

2.4 Zebrafish as a physiological model organism

Zebrafish (*Danio rerio*, previously *Brachydanio rerio*) was found in the river Ganges in early 19th century (Spence et al. 2008). Molecular genetic technologies for the species were established by George Streisinger in the 1980s (Streisinger et al. 1981). It has been gaining popularity as a model organism in behavioural and translational neuroscience during the last decades (Spence et al. 2008). This vertebrate shares similarity with mammals in many aspects, but the low cost, rapid maturation, and ease of genetic manipulation make it a good alternative for many studies. The transparency and robustly distinguishable behavioural patterns at the larval state are useful advantages in physiological and behavioural research.

Zebrafish has also been established as a model for sleep research. Behavioural criteria have been used to define sleep – or sleep-like states – in this diurnal vertebrate (Zhdanova 2006, Chiu & Prober 2013, Elbaz et al. 2013). However, sleep homeostasis has not been clearly demonstrated, especially in larval zebrafish. Light has been earlier reported to overwrite the effect of sleep rebound after sleep deprivation (Zhdanova et al. 2001, Yokogawa et al. 2007, Elbaz et al. 2013, Sigurgeirsson et al. 2013). Our aim in

this study (III) was to verify that zebrafish larvae have homeostatic sleep rebound after sleep deprivation, also in light, to validate this model for sleep research.

After confirming sleep homeostasis, zebrafish is suitable for further studies of the immunological and metabolic findings of our human studies. Zebrafish is a vertebrate model harbouring neuronal systems, immune system, and lipid metabolism that resemble those of humans. Here I briefly review the development, behaviour, and the organs and systems relevant to our current and future research, focusing on the larval stage used in our experiments.

2.4.1 Development

Zebrafish breed all year round in laboratory conditions, produce a large number of offspring, and maturate rapidly. A pair can produce hundreds of fertilised eggs in a single clutch. Embryos hatch from their chorions at 2-3 days post fertilisation (dpf) and are immediately independent (Figure 2). The larvae gain positive buoyancy at 3-4 dpf as the swimming bladder inflates (Spence et al. 2008). By 5 dpf, larvae start to exhibit characteristic movement patterns, including startle responses to external stimuli (Fero et al. 2011, Kalueff et al. 2013). All larvae first develop into females. Sex differentiation begins approximately at 5-7 weeks' age, and sexual maturation of males takes approximately 3 months after which the fish are considered adult. In the wild, the life expectancy of zebrafish is around 1 year, while the mean (max) lifespan in captivity is 3.5 (5.5) years (Gerhard et al. 2002).

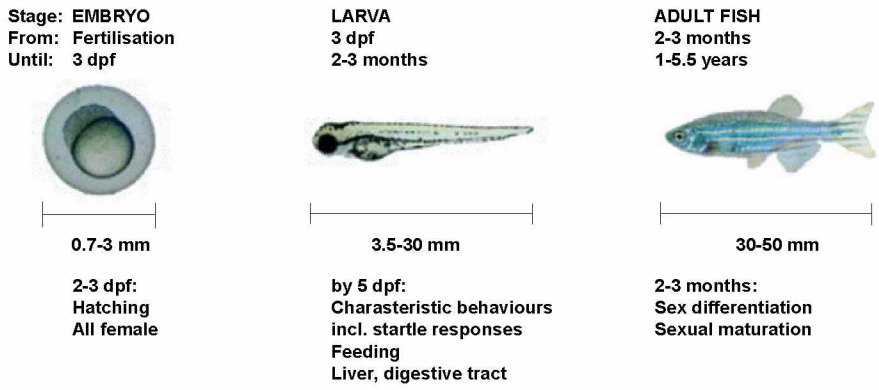

Figure 2. Developmental stages of zebrafish
Zebrafish develop from fertilised eggs to independent larvae in a few days, and to mature adults in a few months. By 5 days post fertilisation (dpf), the larvae have developed characteristic behaviours and learned to feed. Modified from (Alestrom et al. 2006).

2.4.2 Environmental conditions in natural habitat and laboratory

Zebrafish are found in standing ponds and slow-moving streams in the Indian subcontinent (Spence et al. 2008, Parichy 2015). Natural habitat ranges from temperatures between 6-38°C, pH levels ~6-10, and salinities ~0.01-0.8‰ (Spence et al.

2008, Parichy 2015). Optimised living environment in laboratories typically includes a temperature near +28.5 C, pH close to neutral, and salts (e.g. NaCl, KH_2PO_4, $CaCl_2$, $MgSO_4$, and $NaHCO_3$) provided in the swimming water (Westerfield 2000). The natural daylight length ranges from 11 to 14 h per day depending on time of year and location, while in the laboratories zebrafish are typically maintained in a constant light–dark rhythm of 14h/10h (or 12h/12h). The longer light period promotes breeding, which occurs primarily during summertime in the wild (Spence et al. 2008).

Zebrafish are omnivorous, feeding primarily on zooplankton and aquatic insects but also on phytoplankton (Spence et al. 2008). In laboratory conditions, they are usually fed live *Artemia* (brine shrimp) with dry food (with fat content typically 8-15%) (Holtta-Vuori et al. 2010). Feeding behaviour, including prey capture, starts by 5 dpf, but the embryos/larvae can survive with the nutrition provided by the yolk sac until approximately 1 week old (Spence et al. 2008).

2.4.3 Behaviour of the larval zebrafish

Zebrafish larvae hatch at 3 dpf and develop characteristic movement patterns by 5 dpf (Spence et al. 2008). Automatic video recording and movement pattern tracking methods make zebrafish larvae a suitable model for high-throughput analyses of behaviours.

Spontaneous movements of zebrafish larvae include slow swimming, bursts, and routine turns (Budick & O'Malley 2000, Kalueff et al. 2013). In addition, the larvae exhibit escape responses and other responses to their environment, such as the optomotor response (Kalueff et al. 2013). Zebrafish larvae begin feeding behaviour also within a few days after hatching. This includes hunting of live food with complex behaviour for identifying and capturing the prey (Kalueff et al. 2013).

The main predators for zebrafish larvae in their natural habitat are adult fish and dragonfly nymphs (Tabor et al. 2014). To escape from the predators, the larvae respond rapidly to various sensory stimuli – including acoustic, tactile, and visual stimuli – that cross a threshold for initiating an escape response (Fero et al. 2011). Also electrical stimuli have been shown to elicit startle responses, and are often used in laboratory settings (Tabor et al. 2014). A startle response consists of three stages: first the fish contracts its muscles unilaterally to bend the body into a C shape for a fast turning movement, then shows a counter bend, and finally swims rapidly forward (Kalueff et al. 2013) (Figure 3).

In zebrafish larvae, the startle responses are divided into two categories based on the latency of their initiation after the stimulus (Burgess & Granato 2007). The short latency C-start (SLC) begins within 15 ms of the stimulus in normal laboratory temperature (+28.5°C). The movement is characterised as a long latency C-start (LLC) if it is initiated 16-40 ms after the stimulus. A pair of giant reticulospinal neurons, the Mauthner cells, serve as the "command neurons" initiating the SLC (Burgess & Granato 2007). Single firing of one of the Mauthner cells triggers an escape toward the contralateral side

(Nissanov et al. 1990, Korn & Faber 2005). The LLC is not mediated by Mauthner cells, but other reticulospinal neurons sometimes called Mauthner-like cells (Kohashi & Oda 2008).

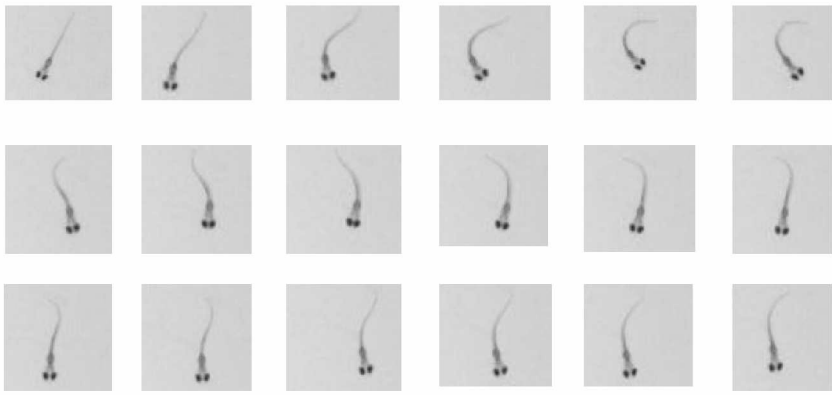

Figure 3. C-start in larval zebrafish
A sequence of high-speed video images (shown 1 image of every 2 ms) illustrating the movement pattern of startle response (starting from top left and proceeding by rows, each from left to right). In response to a startle response-provoking sensory stimulus, a 1-week old zebrafish larva first shows a C-shaped turn, then a counter bend, and finally direct swimming movements.

2.4.4 Genetics

The zebrafish genome has been sequenced and found to have more than 26,000 protein-coding genes (Howe et al. 2013). Of these, 69% have a human orthologue, while 71% of human genes have at least one orthologue in the zebrafish genome (Howe et al. 2013).

As the ancestor of the *teleostei*, the bony fish, underwent a whole-genome duplication event after diverging from the other vertebrae lineages, they have two copies of many genes. The products of this event, called ohnologues (after Susumu Ohno), often have divided the functions of the mammalian homologue (Howe et al. 2013). Thus, in the case of silencing one ohnologue, the other one may at least partly compensate for its function.

Many tools for targeted gene knockdown (morpholino oligonucleotides) and genome-editing (transcription activator-like effector nucleases (TALENs), CRISPR/Cas) are readily available for this animal model (Varshney et al. 2015). Although genetic manipulation is relatively straight forward, special care has to be taken to verify that the studied genes are actually functional orthologues of human genes of interest. Centralised online resources of curated genetic and developmental information for zebrafish researchers are available at the Zebrafish Model Organism Database ZFIN (http://zfin.org) (Ruzicka et al. 2015).

2.4.5 Immune system

The adult zebrafish has both the innate and adaptive immune systems, but a temporal separation in their development offers a possibility to study the vertebrate innate immune response in the larval stage, as the adaptive immune system develops later (mature only after 4-6 weeks) (Novoa & Figueras 2012). Also the maturation of the cells, such as T lymphocytes, can be visualised during the development of the adaptive immune in the transparent larvae (Trede et al. 2004). In addition to the T cells that develop in the thymus, teleost fish like zebrafish have other main players of the adaptive immune system, including B lymphocytes (Trede et al. 2004).

Zebrafish possess the main blood cell lineages found in mammals, including monocytes, macrophages, neutrophils, and lymphoid cells (Galindo-Villegas 2016). Zebrafish also have homologues for interleukins, such as IL-1β, and various other cytokines (e.g. IFN-γ) found in mammals. Mediators of inflammatory signalling cascades, such as toll-like receptors and nuclear factor kappa B (NF-κB), have been found in zebrafish.

2.4.6 Lipid metabolism

Zebrafish can be a useful model in studying lipid-related diseases, such as atherosclerosis (Holtta-Vuori et al. 2010, Asnani & Peterson 2014). The major lipid and lipoprotein classes are conserved between mammals and fish. Zebrafish have white fat tissue, but not brown fat which is associated to homeothermy. Lipids are mostly stored as TAGs in visceral, intramuscular, and subcutaneous adipocyte deposits. Compared to humans, zebrafish have more polyunsaturated lipids. As in mammals, dietary lipids are hydrolysed by lipases, emulsified by bile acids in the gut, absorbed to blood, and transported in triacylglycerol-rich chylomicrons and extra-large VLDLs to the liver.

The liver and digestive tract of zebrafish larvae maturate by 5 dpf (Goessling & Sadler 2015). Liver delivers cholesterol to the extrahepatic tissues, packed in LDL, although this lipoprotein class has been shown to contain more triacylglycerols and less cholesteryl esters than in humans (Holtta-Vuori et al. 2010). Unlike in humans, HDL is the most abundant lipoprotein class in zebrafish (Holtta-Vuori et al. 2010). The main apolipoprotein associated to HDL particles in mammals, apoA-I, is present in zebrafish (Otis et al. 2015). Zebrafish also have the LDL and VLDL-associated apoB (Holtta-Vuori et al. 2010, Otis et al. 2015).

Orthologues for genes coding for cholesterol transporters in humans, such as ABCA1, NPC1, NPC2, NPC1L1, caveolins 1, 2 and 3, and LDL receptor, have been identified in zebrafish (Holtta-Vuori et al. 2010).

Atherosclerotic lesions have been found in many animals in the wild, including poikilothermic animals, such as teleost fish (Holtta-Vuori et al. 2010). The polyunsaturated lipids of fish are susceptible to oxidation, and the modified lipoprotein particles can be cleared by scavenger receptors, as in mammals (Holtta-Vuori et al. 2010). Zebrafish fed with high-cholesterol diet have been shown to have increased

concentrations of LDL and VLDL, lipoprotein oxidation, and fatty streak formation, closely resembling the development of human atherosclerosis (Stoletov et al. 2009).

2.4.7 *In vivo* imaging

Zebrafish embryos and larvae are optically transparent, and thus enable visualisation of (patho)physiological processes in live, behaving animals. As sleep is a behavioural state and function of complex neuronal networks and other systems, most of its functions cannot be studied *in vitro* using cell cultures. Thus, *in vivo* models are needed to study functions of sleep and effects of sleep loss.

With state-of-the-art optical imaging methods, the whole brain or other organs can be monitored with single-cell resolution *in vivo* (Leung et al. 2013). Optogenetic tools can be used to stimulate single cells or neuronal circuits with temporal and spatial precision (Sumbre & de Polavieja 2014). Time-lapse two photon imaging has been used to monitor changes in structural synaptic plasticity, yielding an elegant report on both a circadian and a homeostatic effect on synapses *in vivo* (Appelbaum et al. 2010).

For imaging of lipid metabolism and transport, fluorescent-labelled cholesterol, fatty acids, and phospholipids are readily available (Holtta-Vuori et al. 2008, Holtta-Vuori et al. 2010). In this model, development of lipid deposits in macrophages was visualised for the first time *in vivo* (Stoletov et al. 2009). These methods offer intriguing possibilities for future studies investigating the effects of sleep loss e.g. on cholesterol metabolism and atherosclerotic processes.

2.5 Zebrafish as a model in sleep research

Figure 4. (How) Do zebrafish sleep?
Although zebrafish don't lay down in bed to sleep, they have been reported to have sleep-related place preference and species-specific posture (Zhdanova et al. 2001). Zebrafish don't have eyelids, so they do sleep with their eyes wide open (Árnason et al. 2015). With the current methods, we can only imagine whether zebrafish count seahorses when trying to fall asleep or whether they even dream of seahorses, although fish have been proposed to possess the ability to dream based on their body movements (Corner & Schenck 2015). Printed with the kind permission of the artist Chau Dang (LTD Space).

Do fish sleep? How can sleep be defined without EEG? Aristotle used the study of sleep in fish as an example of biological reasoning. He observed the behaviour of fish and suggested that they indeed do sleep, as they have periods of immobility, deadened to outside stimuli, with specific postures and place preference. He also speculated that there should be a reason for the fish to need to sleep – as they practise it although it poses a threat to them exposing them more susceptible to predation – and pondered whether the function of sleep was analogical in fish to that of humans and other animals.

The attributes defined by Aristotle closely resemble the behavioural criteria Campbell & Tobler proposed in the 1980s to define sleep in animals without EEG (Campbell & Tobler 1984) (Table 1). These criteria have been used to characterise sleep or sleep-like states in many different animal species, including fish, reptiles, worms, and flies (Campbell & Tobler 1984, Zhdanova 2006, Raizen & Zimmerman 2011).

Both adult and larval zebrafish have been reported to have reversible behavioural quiescence periods that can be classified as sleep or sleep-like states (Yokogawa et al. 2007, Zhdanova 2011, Chiu & Prober 2013). Of the two processes regulating sleep and wakefulness, the circadian process has been more extensively studied thus far (Vatine et al. 2011). Studies of the homeostatic process have also been reported, but the presence

of sleep rebound (in light) has not been clearly demonstrated (Chiu & Prober 2013, Sigurgeirsson et al. 2013).

Table 1. Behavioural criteria for sleep
Sleep or sleep-like states can be characterised in animals without EEG/polysomnography using behavioural criteria. The main criteria are marked with bold text. All of the criteria have been reported in zebrafish, but the homeostatic sleep rebound has been shown only in dark conditions. (The References column does not contain an exhaustive list of all evidence backing the fulfilment of these criteria in zebrafish.)

Criterion	References
Spontaneous immobility	(Hurd et al. 1998, Zhdanova et al. 2001, Prober et al. 2006)
Elevated arousal threshold (reduced sensory responsiveness)	(Zhdanova et al. 2001, Prober et al. 2006, Yokogawa et al. 2007)
Reversible (animal can be awakened with a stimulus crossing the threshold)	(Zhdanova et al. 2001, Prober et al. 2006, Yokogawa et al. 2007)
Homeostatic regulation (sleep rebound after prolonged wakefulness)	(Zhdanova et al. 2001, Yokogawa et al. 2007)
Circadian rhythm	(Hurd et al. 1998, Zhdanova et al. 2001, Prober et al. 2006)
Species-specific posture	(Zhdanova et al. 2001, Yokogawa et al. 2007)
Place preference	(Zhdanova et al. 2001, Yokogawa et al. 2007)
(Pharmacological modulation)	(Zhdanova et al. 2001, Rihel et al. 2010a, Sigurgeirsson et al. 2011)

2.5.1 Circadian process

Zebrafish raised in light–dark cycle have been shown to be more active during the day, and have more immobility during the night from 4 dpf onward (Hurd et al. 1998). Circadian oscillations in locomotor activity are maintained even if the fish or larva is moved to constant darkness or constant dim light (Hurd et al. 1998, Elbaz et al. 2013). However, constant light seems to activate zebrafish leading to complete loss of circadian rhythms (Elbaz et al. 2013).

The core molecular machinery of the mammalian circadian clock, such as the Clock, Bmal, Period, and Cry genes, is conserved in zebrafish (Vatine et al. 2011). Yet, zebrafish have several copies of some of these genes, partly due to the genome duplication (Vatine et al. 2011).

Unlike in mammals, light can directly entrain the peripheral clocks in zebrafish, possibly due to the transparency of this species (Chiu & Prober 2013). Thus, it has been hypothesised that zebrafish would not necessarily demand a central "master clock" to synchronise the physiological functions throughout its body (Moore & Whitmore 2014).

Suprachiasmatic nucleus, the mammalian central pacemaker, has been anatomically defined in the zebrafish brain, but it has been suggested not to be required for circadian rhythms (Moore & Whitmore 2014). However, zebrafish do have a pineal gland that transduces light information from the retina into a neural and neuroendocrine (melatonin) signal (Vatine et al. 2011). Resembling the effect in humans, melatonin has a somnogenic effect in zebrafish (Zhdanova 2011).

Circadian rhythms have been detected in various measures, such as the expression of the neuronal activity marker c-fos, regulation of the cell cycle, and structural synaptic plasticity, in zebrafish (Vatine et al. 2011, Elbaz et al. 2013, Moore & Whitmore 2014).

2.5.2 Homeostatic process

Edwin Land has famously noted that "a problem well defined is half solved". Yet, criteria for defining sleep-like states in zebrafish seem to vary, lacking consistency between studies and laboratories.

The main criteria to distinguish sleep from quiet wakefulness are reduced sensory responsiveness and homeostatic regulation (Campbell & Tobler 1984) (Table 1). Increases in arousal threshold have been reported during locomotor inactivity periods in zebrafish (Zhdanova et al. 2001). Prober et al. defined sleep in larval zebrafish as a period of at least 1 minute of inactivity, as they found these rest bouts to be associated with increased arousal threshold (Prober et al. 2006). This definition has been used in their subsequent studies (Rihel et al. 2010b). Yokogawa et al. defined sleep in adult zebrafish as inactivity over 6 s (Yokogawa et al. 2007). Karlsson et al. have used 6 s immobility also for larval zebrafish (Sigurgeirsson et al. 2011, Sorribes et al. 2013).

In addition, changes in respiration and observations of species-specific postures and place preference – floating with head down or staying horizontal close to the bottom – have been reported with prolonged periods of immobility (2-10 min) (Zhdanova et al. 2001, Zhdanova 2006). A study comparing the ontogeny of sleep in zebrafish and humans has reported similarities, with highest sleep amount in younger animals and wake bouts gradually consolidating with age (Sorribes et al. 2013).

Homeostatic regulation of sleep has been assessed in larval (Zhdanova et al. 2001) and adult zebrafish (Yokogawa et al. 2007). However, sleep rebound has not been observed if the fish were let to recover with lights on (Yokogawa et al. 2007, Chiu & Prober 2013). Zebrafish have even been proposed to not have sleep homeostasis (Rial et al. 2007). However, the consensus seems to lie on the hypothesis that they do have sleep homeostasis, but it may be overwritten by the activating effect of light (Chiu & Prober 2013, Sigurgeirsson et al. 2013). Verification of sleep homeostasis in larval zebrafish is crucial before continuing into further studies of sleep regulation and the effects of sleep loss using this model.

2.5.3 Brain systems regulating sleep and wakefulness

Fish lack a developed neocortex, but have many brain regions functionally resembling those of mammals. Structures with cortex-like functions have also been reported (Mueller et al. 2011). Neurotransmitters involved in the regulation of sleep and wakefulness in mammals are conserved in zebrafish (Chiu & Prober 2013).

In mammals, the hypothalamo-pituitary-adrenal axis (HPA axis) links the nervous system to the endocrine system, releasing hormones involved in the regulation of many bodily processes, such as stress, immune system, appetite, and sleep. The aminergic systems in the brainstem and posterior hypothalamus, namely histaminergic, dopaminergic, noradrenergic, and serotonergic system, promote wakefulness (Saper et al. 2005) (Figure 5 A). Together with the hypocretinergic/orexinergic system and the cholinergic system of the brain stem and basal forebrain they form the ascending arousal system that activates cortex and thalamus (Saper et al. 2005).

In zebrafish, hypothalamus is also located in the proximity of the thalamus and connected to the pituitary (hypophysis) (Machluf et al. 2011). Equivalents of the mammalian neuronal nuclei involved in arousal have been identified in the zebrafish brain (Machluf et al. 2011, Chiu & Prober 2013) (Figure 5 B). Of the neurotransmitters, the hypocretinergic system has been studied most intensely regarding sleep and wakefulness in zebrafish (Elbaz et al. 2013). Also the histaminergic system has been shown to be involved in arousal in zebrafish (Sundvik et al. 2011).

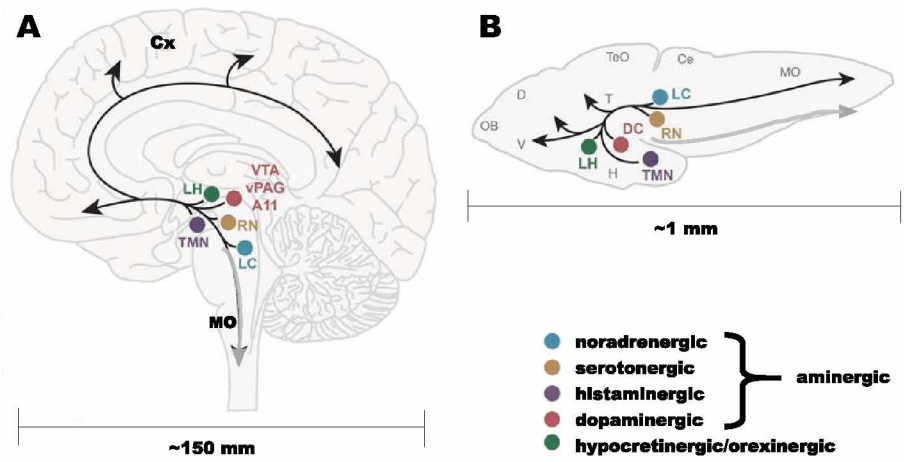

Figure 5. Arousal-promoting neuromodulatory systems in vertebrates
Key neurotransmitter systems that promote arousal in **A)** human and **B)** larval zebrafish brain. Ascending projections (black arrows) increase excitation in the forebrain, while descending projections (grey arrows) promote sensorimotor function and muscle tone. The arousal nuclei inhibit the sleep-active ventrolateral preoptic area (VLPO), which in turn has inhibiting projections on the arousal-promoting neurons, at least in mammals.

Neuromodulatory regions: noradrenergic locus coeruleus (LC), serotonergic raphe nuclei (RN), histaminergic tuberomammillary nucleus (TMN), dopaminergic ventral tegmental area (VTA), ventral periaqueductal grey (vPAG), and mammalian dopamine cell group A11 (A11), diencephalic cluster (DC), and hypocretinergic lateral hypothalamus (LH). Anatomical regions: cortex (Cx), medulla oblongata (MO), olfactory bulb (OB), dorsal telencephalon (D), ventral telencephalon (V), optic tectum (TeO), hypothalamus (H), thalamus (T), cerebellum (Ce). Modified from (Chiu & Prober 2013).

Sleep-active neurons reside in the ventrolateral preoptic nucleus (VLPO) in the mammalian anterior hypothalamus (Saper et al. 2005). These inhibitory GABAergic and galaninergic neurons inhibit the arousal systems, and *vice versa*, creating a "flip-flow switch" type control of sleep and wake (Saper et al. 2005). A recent study in zebrafish reported that a small group of hypothalamic neurons expressing a neuropeptide named QRFP promote sleep and inhibit locomotor activity (Chen et al. 2016).

Also pharmacological experiments suggest that the regulation of sleep and arousal is conserved. Several arousal state-modifying substances from mammals have been found to promote arousal also in zebrafish (Chiu & Prober 2013, Richter et al. 2014). Hypocretin increases arousal and inhibits rest in zebrafish larvae (Prober et al. 2006, Chiu & Prober 2013). Modafinil has been shown to increase wakefulness by lengthening wake bouts in zebrafish similarly to the effect in mammals (Sigurgeirsson et al. 2011).

Also somnogenic effects have been reported. Melatonin, secreted by the pineal gland also in zebrafish, promotes sleep and mediates circadian output (Zhdanova et al. 2001, Gandhi et al. 2015). Galanin has been suggested to have potential sedating functions

(Richter et al. 2014). In zebrafish, overexpression of the *galanin* gene has been shown to decrease spontaneous locomotor activity and reduce responsiveness to sensory stimuli (Richter et al. 2014). However, it is relevant to keep in mind that the definitions for sleep in these studies vary and are mostly based on locomotor inactivity.

The adenosinergic system is known to participate in sleep homeostasis in mammals (Porkka-Heiskanen et al. 1997). Adenosine has been shown to increase in the basal forebrain in sleep deprivation, inhibiting wake-promoting cholinergic neurons via the inhibitory A1 receptor (Porkka-Heiskanen & Kalinchuk 2011). Adenosine receptor agonists have been shown to increase sleep, while antagonists, such as caffeine, promote wakefulness in many species (Landolt 2008, Bjorness et al. 2009). By the time this study was initiated, no reports on the effects of adenosine agonists or antagonists in zebrafish had been published. More recently, a high-throughput drug screen reported that adenosine agonists increased rest while antagonists increased waking activity and reduced rest in larval zebrafish (Rihel et al. 2010a). Adenosinergic signalling was also recently suggested to integrate the homeostatic and circadian regulation of sleep in zebrafish by mediating the sleep-inducing effects of melatonin (Gandhi et al. 2015).

These findings together suggest that the regulatory systems controlling sleep and wakefulness are well-conserved in the brains of vertebrae. However, sleep homeostasis in this model should be verified. Altogether, zebrafish is a useful model in gaining more information on the aspects of sleep in vertebrae, including humans.

3 Aims of the Study

Epidemiological studies have shown that individuals who sleep less have higher risk for cardiovascular diseases. The mechanisms are not fully understood. The general aim of this study was to explore pathways affected by sleep loss that may participate in the development of atherosclerosis, the underlying pathological process for cardiovascular events such as myocardial infarction and stroke.

The development of atherosclerosis involves inflammatory activation and altered metabolism. Cholesterol accumulation in tissues is a crucial part in the development of atherosclerosis. Chronic low-grade inflammation also plays a major role in this process.

I hypothesized that sleep loss alters cholesterol metabolism, possibly via immune system activation (Figure 6). Both the inflammatory activation and changes in cholesterol metabolism could participate in increasing the risk for atherosclerosis. Further studies of these mechanisms require animal models. I hypothesized that zebrafish larvae do exhibit a homeostatic sleep rebound that can be detected using suitable methods for sleep deprivation and detection. After confirming sleep homeostasis in larval zebrafish, they may serve as a useful model for studying the findings of the human studies.

The specific aims were

I. to elucidate the effects of sleep loss on immune functions at the level of gene expression in leukocytes
II. to evaluate the effects of sleep loss on lipid metabolism and transport
III. to develop and validate a diurnal vertebrate model for further studying the mechanisms of the effects of sleep loss on immune functions and metabolism

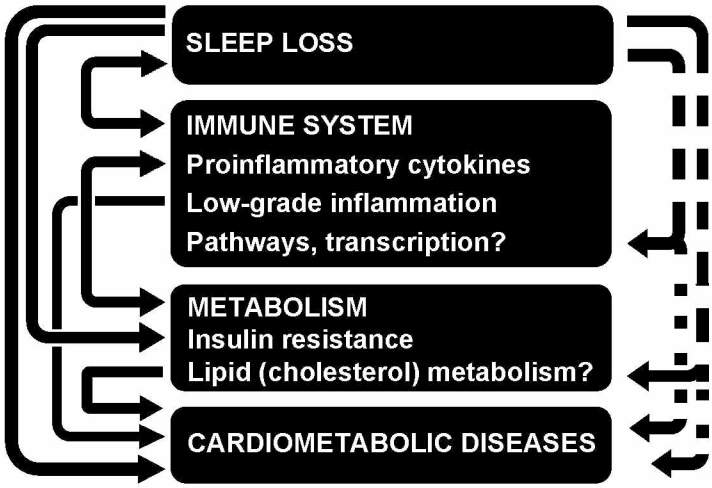

Figure 6. Interconnections between sleep, immune functions, and metabolism
Epidemiological studies have suggested an association between sleep loss and cardiometabolic diseases, such as atherosclerosis and type II diabetes. Inflammation and metabolic dysregulation are in the centre of the development of these diseases. Sleep is known to be tightly interconnected with the immune system; sleep loss increases proinflammatory cytokines, which in turn increase NREM sleep. Sleep loss has also been shown to drive carbohydrate metabolism to the direction of insulin resistance, but the effects on lipid metabolism are not clear. In this thesis, I seek to elucidate immune system and cholesterol metabolism-related pathways affected by sleep loss on the transcriptional level, focusing on changes that may contribute to the development of cardiometabolic diseases, especially atherosclerosis (dashed arrows on the right). I use experimental sleep restriction to study the effects of sleep loss in carefully controlled laboratory conditions, and epidemiological cohorts to estimate the effects of sleep loss in real-life conditions.

4 Materials and Methods

The effects of sleep loss on immune functions and cholesterol metabolism in humans were studied in both experimental sleep restriction and epidemiological cohorts. Gene pathways affected by sleep loss were assessed using transcriptomics. Changes in serum metabolites were measured with nuclear magnetic resonance (NMR) metabolomics. A system for sleep-depriving zebrafish larvae was developed for future studies on the mechanisms behind the changes observed in the human studies.

4.1 Human samples

A laboratory experiment simulating a working week with restricted sleep was conducted to study the effects of sleep loss in highly controlled laboratory conditions. To estimate the effects in real life, two Finnish epidemiological cohorts were used.

4.1.1 Experimental sleep restriction (SR) study

Sleep of healthy young male volunteers (N=14) was restricted to 4 h/night for 5 consecutive nights (sleep restriction, SR) (Figure 7). Control subjects were given 8 h/night time in bed (N=8 originally, decreased to 7 as one control subject failed to sleep sufficiently in the laboratory). All subjects spent 8 h in bed for the first 2 nights (baseline, BL). For the biomedical analyses, venous blood samples were taken after overnight fasting at BL and after 5 nights of SR.

All subjects reported a habitual sleep duration of 7-9 h, with habitual bedtime before midnight and wake-up time after 6 a.m. The participants were screened prior to the experiment, and individuals with extreme circadian types, sleep disorders, potential jetlag, habitual daytime napping, shift work, a history of drug or alcohol dependence, chronic or recent acute medical conditions, or psychiatric illness were excluded. All subjects were healthy young males of 19-29 years of age (mean±s.d. 23.2±2.2 years).

BMI was 23.5 (±2.6) in the sleep restriction group and 23.2 (±2.4) in the control group before the experiment. Meals were standardised using the national nutrition recommendations (Finnish nutrition recommendations – balancing energy intake and energy expenditure. 2005). An additional piece of fruit was given to the sleep restriction group on the SR nights. The participants were not allowed to consume caffeine, alcohol, or tobacco during the experiment.

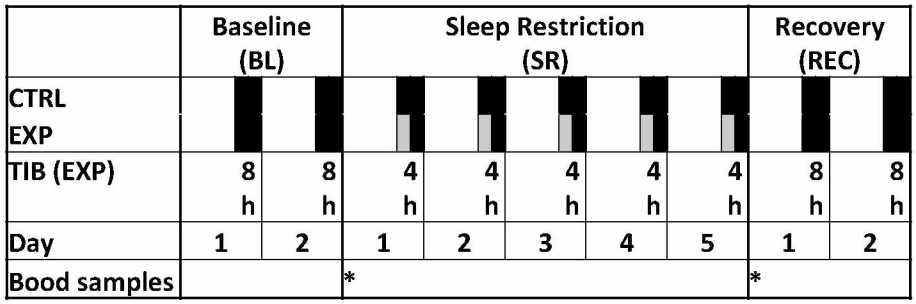

Figure 7. Experimental sleep restriction (SR) protocol
After a baseline (BL) of 2 nights, the experimental (EXP) group had 5 nights of 4 h (03-07, black) sleep opportunity per night, while the control (CTRL) group had 8 h (23-07) time in bed (TIB) during the whole experiment. Fasting blood samples (*) drawn in the morning after the SR period were compared to those taken after the BL.

4.1.2 DILGOM cohort

To assess the effects of sleep loss in real-life conditions, a subsample of the FINRISK 2007 population cohort was used. FINRISK cohorts are Finnish representative, cross-sectional population surveys. The sample collections have been carried out every 5 years starting from 1972 to study health behaviours in the working age population (age range 25-74 years) and risk factors of chronic diseases, such as cardiovascular diseases and cancer. The cohorts comprise laboratory measurement data from fasting blood samples in addition to questionnaire information on socioeconomic status, medical history, and lifestyle.

The Dietary, Lifestyle, and Genetic determinants of Obesity and Metabolic syndrome (DILGOM) is a subsample of FINRISK 2007 with more information to characterise risk factors for metabolic and cardiovascular diseases in the Finnish population (Inouye et al. 2010a). For a subsample from the Helsinki metropolitan area (N=518; mean±s.d. age 51.9±13.8 years; 54% females), the cohort includes datasets of transcriptomics and NMR metabolomics (Inouye et al. 2010b).

The sufficiency of sleep was estimated using the question "Do you, in your opinion, sleep enough?" with four answer options: 1) "Yes, almost always" (N=168), 2) "Yes, often" (N=218), 3) "Seldom or almost never" (N=86), and 4) "I cannot say" (N=46). Subjects reporting to sleep enough "seldom or almost never" (N = 86) were classified as having 'subjective sleep insufficiency' (SSI; N = 86; 18.2%), and compared to the subjects reporting to "often" or "almost always" sleep enough (noSSI; N = 386). Habitual sleep duration was reported in hours.

4.1.3 Young Finns Study (YFS) cohort

To replicate the results in an independent cohort, the 2007 time point of the Cardiovascular Risk in Young Finns study (Young Finns Study, YFS; N=2221; mean±s.d. age 37.7±5.0 years; 55% men) was used. This Finnish longitudinal sample

collection was initiated in 1980 to study the risk factors and precursors of cardiovascular diseases and their determinants in children and adolescents (Raitakari et al. 2008). The participants were 30-45 years old in the follow-up time point of 2007 used in this study. Fasting blood samples have been drawn for biomedical analyses, and questionnaire information obtained on e.g. various lifestyle factors.

Questions on self-reported sleep need and duration were used to estimate the sufficiency of sleep. The self-reported sleep duration was subtracted from the self-reported sleep need of each individual, and participants were grouped into four groups: heavy SSI (hSSI; sleep need – sleep duration > 2 h; N = 55, 2.5%), moderate SSI (mSSI; 1.5–2 h; N = 304, 13.7%), no (or only mild) SSI (noSSI; −1…0…1 h; N = 1825, 82.2%), and oversleep (OS; < -1 h; N=37, 1.7%; excluded from further analyses).

Probable self-reported obstructive sleep apnoea (OSA) was defined using questionnaire information on the quality and frequency of snoring and respiratory pauses, as described earlier in (Niiranen et al. 2015). A prevalence of 5.2% for probable self-reported OSA was found in this sample using these criteria (Study II). As expected, subjects with OSA-indicating symptoms reported more SSI (probable self-reported OSA prevalence was 4.6% in the noSSI group, 7.8% with mSSI, and 13.6% with hSSI).

4.2 Gene expression

Genes carry the hereditary information in living organisms. This information is coded in the base sequence of DNA molecules, packed into chromosomes. Genes could be thought of as recipes to cook proteins, which then act in the various processes of the body. Although virtually all cells of an individual contain the same genes throughout life, genes can be more or less "active" in different situations.

One way to find out which processes are affected by e.g. sleep loss, is to measure which genes are more or less active in subjects with sleep loss than in baseline conditions or control subjects with sufficient sleep. This can be done to one or a few genes of interest, using e.g. quantitative polymerase chain reaction (qPCR). With modern high-throughput methods, all the tens of thousands of genes in e.g. a human white blood cell sample can be analysed simultaneously on a microchip. These kind of techniques are called *omics* methods. When the expression of all genes is analysed, the method is called *transcriptomics* (as the passing of the genetic information from gene "recipe" to mRNA is termed *transcription*). Similarly, if genetic information is assessed, it is *genomics*, while high-throughput measurements of metabolites, protein, and lipids are called *metabolomics*, *proteomics*, and *lipidomics*.

4.2.1 Microarrays

Transcriptomics was applied to study which genes were less or more expressed in sleep loss. Genome-wide expression profiles were measured from circulating blood cells using DNA microarrays.

In the experimental SR study, peripheral blood mononuclear cells were isolated with Ficoll density gradient centrifugation, and total RNA was extracted using acid guanidinium thiocyanate–phenol–chloroform extraction. Gene expression was measured with Affymetrix GeneChip Human Genome U133 Plus 2.0 arrays. 15,101 probe sets (for genes or transcripts; from here on called simply 'genes') passed the quality control steps. The next filtering step yielded 2,331 genes with at least 1.2-fold change (FC) from BL to SR. A rank-based pathway analysis was run on these sets of 1,292 up-regulated and 1,039 down-regulated genes. The effect of SR on gene expression was estimated using 2-way repeated measures ANOVA (with group as the between-subjects factor and time point as the within-subject factor) followed by paired *t* tests between the time points (Study I).

In the epidemiological cohorts, total RNA was extracted from peripheral blood samples using a centrifuge protocol with proteinase and DNase. Gene expression was assessed using Illumina HumanHT-12 Expression BeadChips and data analysis was conducted as described in (Inouye et al. 2010b, Raitoharju et al. 2014) (Study II).

Probes passing the quality control (N=35,420) and subjects with microarray and sleep sufficiency data (N=472) were included in the DILGOM analysis of differentially expressed genes. Linear regression adjusted for age and gender was used to compare subjects with SSI to those that reported sufficient sleep. 725 genes had lower expression among subjects with SSI (N=86) compared to those with no SSI (N=386) (pointwise $P<0.05$). The biological processes enriched among these gene sets were assessed in the pathway analysis (Study II).

In the YFS replication cohort, the expression of 4 lipid metabolism-related genes discovered in the pathway analysis of the DILGOM sample was assessed in 1,407 subjects (with microarray and sleep sufficiency data). A similar linear regression analysis for the effect of SSI, adjusting for age and sex, was used as for the DILGOM sample (Study II).

4.2.2 Pathway analysis

To elucidate the biological processes affected by sleep loss, pathway analyses using Gene Ontology (GO) classification of genes were performed. These analyses test whether there is an enrichment of genes annotated to certain biological functions among the genes with higher or lower expression in a given condition, e.g. sleep loss.

In the experimental SR study, an in-house developed, rank-based pathway analysis program was used (Pietilainen et al. 2008) (Study I). In the DILGOM cohort, pathway analysis was conducted using Database for Annotation, Visualization and Integrated Discovery (DAVID Bioinformatics Resources 6.7, NIAID/NIH, http://david.abcc.ncifcrf.gov/home.jsp) (Huang da et al. 2009) (Study II). Due to the hierarchical nature of GO annotations, the GO pathways were grouped using DAVID Functional Annotation Clustering, yielding clusters of functionally similar pathways

instead of individual pathways. The pathway analyses in all three data sets were performed separately for the gene sets up- and down-regulated by sleep loss to discover activated and inactivated biological processes.

4.2.3 Transcription factors

To study which transcription factors may have been involved in the differential regulation of gene expression, a transcription factor binding site analysis was conducted in the experimental SR study. Over-representation of binding sites for specific transcription factors within the genes with changed expression after SR was analysed using oPOSSUM (Ho Sui et al. 2005). As for the pathway analyses, up- and down-regulated gene sets were analysed separately. Over-representation of binding sequences within 5.000 base pairs up- or downstream from the transcription start site of each gene was statistically estimated using one-tailed Fisher exact probability test (Study I).

4.2.4 Quantitative PCR

To verify the microarray detection of gene expression in the experimental SR study, the expression of six genes from the same sleep-restricted subjects was analysed also with qPCR. The relative differences in expression between SR and BL time points were using the ΔΔCt method with *PPIA* and *RPLP0* genes as internal controls ("housekeeping genes"). Paired *t* tests were used to compare the expression of each of these six genes, *BTG2*, *FCRL2*, *HIPK3*, *IKZF1*, *STX16*, and *TGFBR3*, between the SR and BL time points (Study I).

4.3 Metabolism

4.3.1 NMR metabolomics

Metabolites in body fluids, such as blood, reflect multiple physiological and pathophysiological processes. A proton nuclear magnetic resonance (NMR)-based metabolomics method was applied to assess effects of sleep loss on serum metabolites (Soininen et al. 2015). This high-throughput platform distinguishes 14 subclasses of lipoproteins (chylomicrons, 5 VLDLs, intermediate density lipoprotein (IDL), 3 LDLs, and 4 HDLs) based on their size. In addition, concentrations of cholesterol, various fatty acids, amino acids, and low molecular weight energy metabolites are measured. The molecules are recognised spectroscopically based on the resonance of their proton (^{1}H) nuclei in a magnetic field. The details of the analysis have been described previously (Soininen et al. 2009, Inouye et al. 2010a). The statistical analysis was conducted with 2-way repeated measures ANOVA and paired *t* tests in the experimental SR study, and linear regression adjusting for age and sex in the epidemiological cohorts (Study II).

4.3.2 Lipid transfer proteins

To detect whether changes in lipoprotein particle concentrations and compositions were mediated via lipid transfer proteins, the activity of PLTP and CETP was measured using liquid scintillation counting in serum samples from the experimental SR study. PLTP activity was assessed with phosphatidylcholine liposomes (Jauhiainen et al. 1993).

Transfer of radiolabelled [14C]cholesteryl oleate between exogenously added human LDL and HDL was measured to estimate CETP activity (Groener et al. 1986). Changes in the activities were estimated statistically with 2-way repeated measures ANOVA (with group as the between-subjects factor and time point as the within-subject factor) and paired *t* tests (comparing SR to BL) (Study II).

4.3.3 Enzymes

Activity of LCAT in serum samples from the experimental SR study was measured as the cholesterol esterification activity using exogenous [3H]cholesterol-labelled HDL proteoliposome discs (Jauhiainen & Dolphin 1986, Kleemola et al. 2002). PON1 activity in serum was assessed spectroscopically using diethyl-p-nitrophenyl phosphate as the substrate (Kleemola et al. 2002). The statistical analysis was conducted similarly as for the lipid transfer proteins (Study II).

4.4 Zebrafish

To develop a model for further studying the findings from the human studies, rest homeostasis was studied in zebrafish larvae. To confirm rest homeostasis in this model, a system for rest-depriving larval zebrafish and quantifying their responses to sensory stimuli was developed (Study III).

4.4.1 Animals and housing

Wild type zebrafish larvae were raised in embryonal medium E3 with pH 7.2. This medium provides 5.00 mM NaCl, 0.44 mM CaCl2, 0.33 mM MgSO4 and 0.17 mM KCl in the swimming water. The "Turku" strain has been maintained in the laboratory for a couple of decades and used for various studies (Sundvik et al. 2013).

Water temperature was kept at +28.5°C±0.5°C, and light-dark cycle at 14:10 with lights on at 9:00 (Zeitgeber Time (ZT)0) and off at 23:00 (ZT14). The larvae were fed with commercial SDS-100 fry food from 4-5 dpf, and used in the experiments starting from 7 dpf. Sex differentiation had not occurred at the time of the experiments (Study III).

4.4.2 Rest deprivation

To confirm rest homeostasis in zebrafish, I designed a system for rest-depriving zebrafish larvae with a naturalistic stimulus, flow of water (Figure 8). Briefly, eighty 1 week old larvae were randomly divided into rest-deprived (RD, N=40) and control (N=40) groups. RD larvae swam against a flow of water for 6 hours during night-time in the dark (23-05) (Figure 9), while control larvae stayed in similar conditions but without the rest-depriving water flow (Study III).

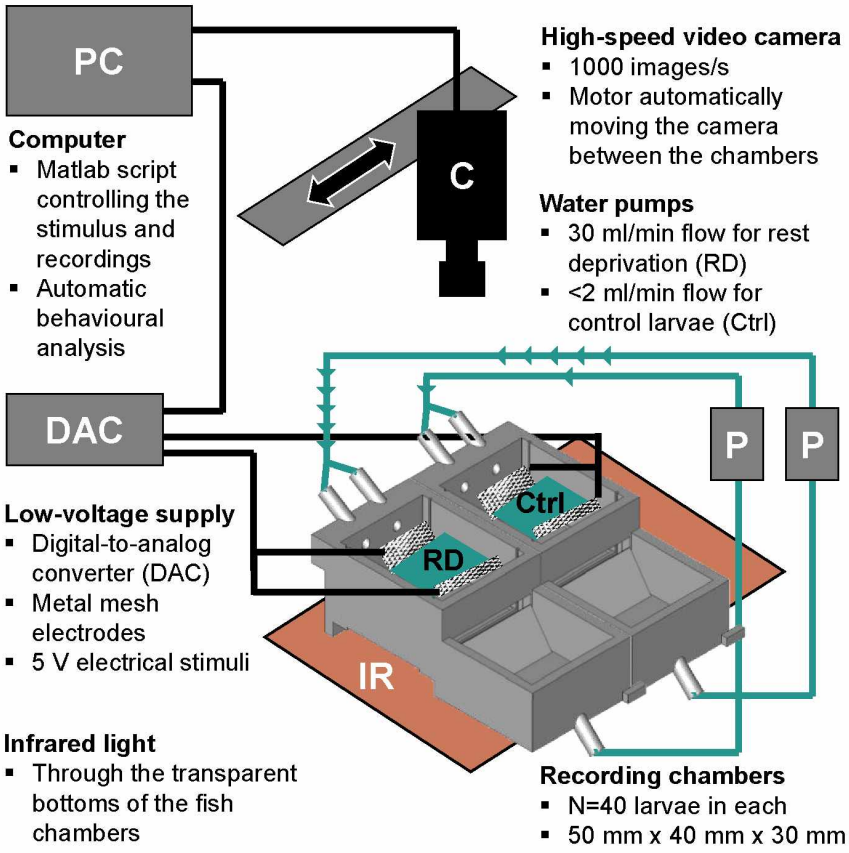

Figure 8. System for rest-depriving zebrafish larvae and testing their responses to sensory stimulation
A system was designed for rest-depriving zebrafish larvae with a naturalistic method, flow of water. To test sensory responses after the rest deprivation, escape reactions after electrical stimuli were recorded using a high speed video camera. Startle responses with short or long latency were detected with software designed for this purpose by (Burgess & Granato 2007). Modified from Study III.

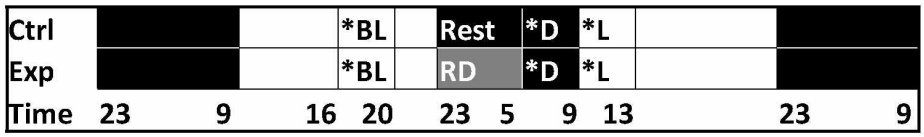

Figure 9. Rest deprivation schedule for zebrafish larvae
Baseline (BL) recordings of startle responses elicited by electric stimuli were conducted in the afternoon. The experimental (EXP) group larvae were rest-deprived 6 h from the beginning of the dark phase (23-05). Startle responses were recorded during the remaining of the dark phase (05-09), and again in the beginning of the light phase (09-13). All larvae were kept in 14h:10h light–dark rhythm throughout the experiment. Infrared lighting was used for the recordings.

4.4.3 Startle response

Escape responses provoked by sensory stimuli in zebrafish occur in a scale of milliseconds (Figure 3). To test whether rest-deprived larvae are less likely to respond to sensory stimuli, I developed a system for electrical stimulation (Figure 8). The responses were recorded with a high speed video camera. The recording rate was set to 1000 images/s and 200 ms was recorded after each stimulus to observe the short latency C-starts (SLC) occurring within 15 ms of the stimulus and the long latency C-starts beginning 16-40 ms after the stimulus. 40 larvae were stimulated and recorded simultaneously in the recording chamber. The behavioural analysis was conducted automatically in batches using software specifically developed for this purpose by Harold A. Burgess and Michael Granato and described in (Burgess & Granato 2007). First, the movements of the larvae were tracked with Flote software, and then the tracking files were analysed with Batchan software to distinguish SLCs and LLCs from other movements (Study III).

4.4.4 Adenosinergic system

To test whether adenosine could increase sleep-like behaviours in zebrafish larvae, selective adenosine receptor A1 agonist CHA was administered in the swimming water (final concentration 100 µmol/l). Startle responses in response to electrical stimuli were tested after CHA administration. Inactivity bouts were quantified after CHA and adenosine antagonist caffeine (100 µmol/l) administration (see details in Supplemental Methods and Figures in Study III). Inactivity periods lasting longer than 60 s were defined as sleep, based on previous studies (Prober et al. 2006, Rihel et al. 2010a, Rihel et al. 2010b).

4.5 Ethical permissions

The experimental SR study was approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa. The cohort studies were approved by ethical committees of the hospital districts. An informed consent was obtained from all participants, and the studies were conducted in accordance with the Declaration of Helsinki (studies I and II).

The zebrafish experiments were approved by the Regional State Administrative Agency for Southern Finland and the National Animal Experiment Board of Finland in agreement with the ethical guidelines of the European Convention (Study III).

5 Results and Discussion

5.1 Experimental and epidemiological sleep loss

Experimental studies in controlled conditions and epidemiological studies in population cohorts can complement each other. A workshop with a focus on the impact of sleep and circadian disruption on energy balance and diabetes held in 2015 stated that "Studies in humans need to complement the elegant short-term laboratory-based human studies of simulated short sleep and shift work etc. with studies in subjects in the general population with these disorders" (Arble et al. 2015). In this thesis, I investigated the effects of sleep loss both in experimental conditions and population samples. By these means, it is possible to obtain information of processes affected in controlled conditions and test whether similar effects occur in real-life conditions.

5.1.1 Cumulative sleep restriction

The sleep restriction experiment was earlier described in (van Leeuwen et al. 2009, Haavisto et al. 2010, van Leeuwen et al. 2010). Findings on cognitive performance, glucose metabolism, appetite-regulating hormones, and white blood cell characteristics were reported in these previous publications.

Total sleep time decreased during the sleep restriction as planned (Figure 10) (Haavisto et al. 2010). The sleep-restricted group (N=14) had a mean (±s.d.) sleep duration of 7 h 22 min (±20 min) in BL and 3 h 54 min (±5 min) in SR. The amount of light NREM and REM sleep decreased, while deep NREM sleep stayed relatively constant (Mikko Härmä, Tarja Porkka-Heiskanen et al., unpublished results). No major changes were observed in the sleep duration of the control subjects (N=7; total sleep time 7 h 19 min (±16 min) and 7 h 26 min (±17 min) at the same time points) (Study II). Subjective sleepiness assessed with the Karolinska Sleepiness Scale and objective sleepiness measured using EEG/EOG increased, and performance in the psychomotor vigilance task and a cognitive multitasking deteriorated in the sleep-restricted subjects (Haavisto et al. 2010).

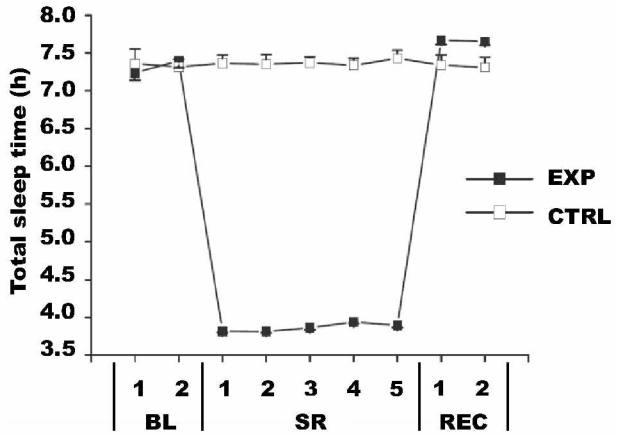

Figure 10. Sleep duration in the sleep restriction (SR) experiment
After an adaptation period of two nights (baseline, BL), the sleep opportunity of subjects in the experimental (EXP) group was restricted to 4 h per night for 5 nights. The total sleep time decreased accordingly to 3 h 54 min (±5 min) in SR. When the participants were let to recover for two nights (REC) with 8 h/night, their sleep increased slightly. Control (CTRL) subjects had 8 h/night time in bed throughout the experiment, of which they slept 7 h 22 min (± 19 min). Modified from (Haavisto et al. 2010).

Of the previously reported physiological measures, immune system was activated in SR (van Leeuwen et al. 2009). Number of B cells increased, while natural killer cells decreased. Production of proinflammatory cytokines IL-1β, IL-6, and IL-17 in response to stimulation was increased in SR. Proinflammatory cytokines, especially IL-1b, tumour necrosis factor alpha (TNF-α), and IL-6, have been consistently shown to increase in experimental sleep restriction and total sleep deprivation also in earlier studies (Mullington et al. 2010, Zielinski & Krueger 2011).

The acute phase protein C-reactive protein (CRP) increased in our experimental SR protocol (van Leeuwen et al. 2009). We also found an association in men – but not in women – with SSI in the epidemiological DILGOM sample (Study I). Males reporting SSI had higher CRP (mean±s.d. 2.61±7.02 mg/l) than males with sufficient sleep (1.92±4.13 mg/l) ($P = 0.0017$). These findings support the activation of the acute phase response and low-grade inflammation in sleep loss.

Experimental sleep restriction has been consistently shown to affect the regulation of glucose metabolism (Spiegel et al. 1999, Depner et al. 2014). Also in our SR experiment, the ratio of insulin to glucose in the blood glucose increased in SR (van Leeuwen et al. 2010). The decrease in insulin sensitivity may in the long run lead to the development of type II diabetes (Depner et al. 2014).

Sleep deprivation has been found to decrease the satiety hormone leptin and increase appetite (Knutson et al. 2007). However, in our SR experiment an increase rather than

decrease in serum leptin was observed (van Leeuwen et al. 2010). No changes in subjective feeling of satiety or BMI were found (van Leeuwen et al. 2010).

5.1.2 Epidemiological sleep insufficiency

In the epidemiological samples, subjective sleep insufficiency was assessed using self-reported questionnaire information. Although the sufficiency of sleep was estimated using different questions, and there were differences also in e.g. the age range, the prevalence of SSI was found to be similar in the two epidemiological samples (Figure 11). Self-reported habitual sleep duration also decreased with increasing level of SSI. In the DILGOM, 18% reported to "seldom or almost never" sleep enough, while in the YFS, 16% had self-reported habitual sleep duration more than an hour shorter than their subjective sleep need (Study II). These findings are roughly in line with an earlier study in French young adults, which reported a 20% prevalence for "sleep debt" using closely similar criteria as the one used in our YFS sample (Leger et al. 2011).

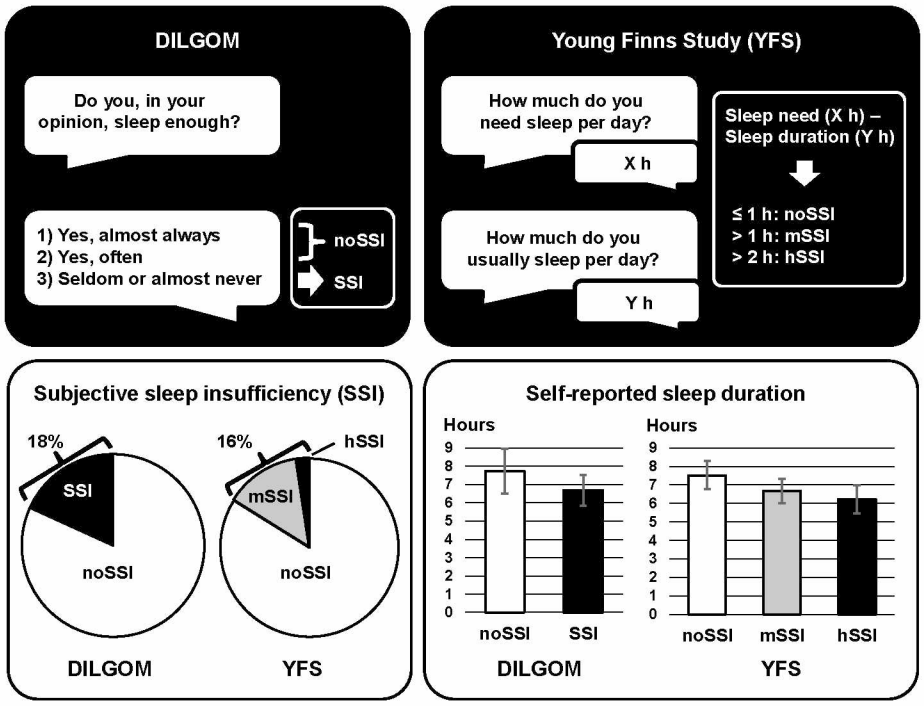

Figure 11. Subjective sleep insufficiency (SSI) in the epidemiological samples
In the epidemiological studies, the sufficiency of sleep was assessed using questionnaire information. In the DILGOM sample, one question addressing the subjective sufficiency of sleep was utilised. In the Young Finns Study (YFS), self-reported sleep duration was subtracted from subjective sleep need to divide the subjects into groups of sufficient sleep (noSSI), moderate SSI (mSSI), and heavy SSI (hSSI). Using these criteria, sleep insufficiency was characterised in 18% and 16% of the subjects in DILGOM and YFS samples, respectively. The mean (±s.d.) sleep duration was shorter in the SSI groups compared to the noSSI groups.

5.1.3 Methodological considerations

In real life conditions, such as shift work, sleep curtailment is often accompanied by circadian misalignment (Moller-Levet et al. 2013). In experimental research, studies can be designed to focus on the homeostatic and/or the circadian process. In our experiment, we simulated the sleep restriction occurring during a busy week (without changing shifts), where wake is typically prolonged by going to sleep later. Prolonged wakefulness has been shown to decrease the amplitude of circadian oscillation and reduce the number of rhythmically expressed transcripts (Maret et al. 2007, Moller-Levet et al. 2013). In this experiment, circadian rhythm, measured as the morning peak in salivary cortisol (Elder et al. 2014), was delayed only 16 min in SR (mean (±s.d). from 07:39 (±0:14) in BL to 07:55 (±0:11) in SR) (van Leeuwen et al. 2010). Thus, we suggest that the observed changes in physiology are mainly caused by the homeostatic sleep curtailment, although the circadian component cannot entirely be ruled out.

Sleep deprivation is often accompanied by stress, which is also associated to many of the measured endpoints along with cardiovascular diseases. Thus, it may be difficult to distinguish which of the effects are caused by the loss of sleep *per se*, and which are effects of e.g. a stressful sleep deprivation protocol. However, the stress hormone cortisol was not elevated in the course of our sleep restriction protocol (van Leeuwen et al. 2009).

In the epidemiological studies, we grouped subjects based on subjective sleep insufficiency instead of sleep duration. An individual is considered to have obtained enough sleep when he/she feels refreshed and fully functional during the day. Sleep loss is considered to occur when an individual gets less sleep than he/she needs. Sleep need is partly genetic, varies between individuals, and can also vary in the same individual e.g. in case of an infection. No objective physiological measure for sleep need or sleep loss has been found thus far. Sleep duration and sleep (in)sufficiency are two independent, although usually highly correlated, aspects of sleep. As Grandner *et al.* have suggested, "short sleep" is not an ideal descriptor for scientific purposes as "it doesn't address how short the sleep is, what the shortness is relative to, and how it was determined" (Grandner et al. 2010). By addressing the sufficiency – instead of duration – of sleep, the natural short sleepers getting enough sleep regarding their subjective sleep need can be distinguished from the subjects not getting sufficient sleep. Some studies on natural short sleepers have proposed that they have fundamental differences in sleep homeostasis and circadian rhythms (Aeschbach et al. 2001, Grandner et al. 2010). Thus, a phenotype of SSI may provide even more information on the actual effects of sleep loss than the mere sleep duration.

Subjective measures in general may not be as reliable as objective measures. E.g. subjective sleep duration has been found to often be over-reported compared to e.g. actigraphy-measured sleep duration, especially by individuals with shorter sleep durations (Lauderdale et al. 2008). Other health-related and sociodemographic factors can also affect the self-reporting (Lauderdale et al. 2008).

Sleep loss and feeling of insufficient sleep may result from various reasons, e.g. work and leisure time activities, mental or physical illnesses, or sleep problems, such as insomnia, obstructive sleep apnoea (OSA), or narcolepsy. OSA is known to be associated to cardiovascular diseases, inflammation, metabolic alterations, and insufficient sleep (Quercioli et al. 2010, Dong et al. 2013, Niiranen et al. 2015). While the experimental study subjects were examined before the experiment to not have any sleep disorders or other medical conditions, the epidemiological samples comprised of normal population. Thus, also subjects with chronic diseases, such as OSA, were included. To evaluate whether OSA could explain our results on decreased HDL, an estimate of potential OSA using self-reported symptoms was added in the model (Niiranen et al. 2015). Although subjects reporting symptoms of OSA also reported more insufficient sleep, SSI was independently associated to lower large HDL (Study III).

Also other lifestyle and health aspects can have an effect on the observed differences in the population samples between subjects with SSI compared to those with sufficient sleep. Individuals with subjective sleep insufficiency have been shown to report more poor general health, frequent physical distress, frequent mental distress, activity limitations, depressive symptoms, anxiety, and pain (Strine & Chapman 2005). According to the same study, they were significantly more likely to smoke, to be physically inactive, to be obese, and, among men, to drink heavily. Our findings of decreased cholesterol transport-related gene expression and large HDL were significant also with BMI added in the model, suggesting that obesity does not explain the results. However, not all of the possible confounding factors were studied. E.g. evening chronotype has been studied in regards to sleep sufficiency, cardiovascular measures, and type II diabetes in the FINRISK 2007 sample (Merikanto et al. 2013). Evening types have been found to report more subjective sleep insufficiency in this sample (Merikanto et al. 2012), and could be included in further analyses of the epidemiological data.

5.2 Immune system-related pathways were activated in sleep loss

5.2.1 Transcriptomics

Genome-wide microarray data was filtered to find differentially expressed genes. The filtered gene sets were analysed with pathway analysis to find biological processes affected by sleep loss. The results are briefly reviewed here focusing on potential mediators of atherosclerotic processes.

Pathway analysis showed that several immune response-related biological processes were enriched among the up-regulated genes in the experimental SR study (permuted $P<0.01$; Table 3 in Study I). Up-regulation of the top-ranked pathway, "B cell activation" (permuted $P<0.001$) is in line with the earlier reported finding of increased amount of B cells in this SR experiment (van Leeuwen et al. 2009). B lymphocytes have traditionally been considered atheroprotective, but this concept has been challenged lately (Nilsson

& Fredrikson 2010), and the roles of in atherogenesis are likely to vary between B cell subtypes (Perry et al. 2012).

The next pathways on the up-regulated list, "interleukin-8 production" and "lipopolysaccharide binding" (permuted $P<0.001$) were also related to the activation of the immune system. These pathways comprised of several toll-like receptors (TLR4, TLR8, TLR7, TLR2) among other genes. These receptors, especially TLR4, play a fundamental role in the recognition of pathogen-associated molecular patterns, such as the bacterial lipopolysaccharides (LPS), and endogenous damage associated molecular patterns. Upon binding of exogenous or endogenous ligands, TLR4 triggers a nuclear factor kappa B (NF-κB)-mediated signalling cascade via MYD88 and activates the production of proinflammatory cytokines IL-1β, TNF-α, and IL-6 (Zielinski & Krueger 2011) (Figure 12). "I-kappaB kinase/NF-kappaB cascade" was among the down-regulated pathways (permuted $P<0.005$), including genes coding for MYD88 and caspase 1 (CASP1). CASP1 acts as the activator of IL-1 by proteolytically cleaving its inactive precursor (McGettrick & O'Neill 2013). In addition to their important role in host defence, the proinflammatory processes mediated by these factors are intrinsically involved in the pathogenesis of atherosclerosis in the case of prolonged activation (Hansson & Libby 2006, Zamani et al. 2013).

Figure 12. Sleep loss activates a signalling pathway producing interleukin 1 beta (IL-1β)
Bacterial lipopolysaccharides (LPS) and endogenous damage associated molecule patterns (DAMP) bind to toll-like receptor 4 (TLR4). TLR activation triggers a signalling pathway with MYD88 and nuclear factor kappa B (NF-κB), leading to proinflammatory cytokine production. Caspase 1 cleaves the pro-IL-1β to the biologically active IL-1β. In addition to the TLR4- and NF-κB-mediated pathway, caspase 1-activating NLRP3 inflammasome has also been suggested to be activated by sleep loss (Zielinski & Krueger 2011). Modified from (McGettrick & O'Neill 2013).

NF-κB activation has been reported to increase in sleep deprivation in the white blood cells in humans and in the brain in rodents (Chen et al. 1999, Irwin et al. 2008). To my knowledge, changes in TLR4 had not been reported in humans before (Study I). Recently, another study on healthy males reported increased expression of *TLR4* and *TNFA* after 25 h of total sleep deprivation (Chennaoui et al. 2014), in line with my finding of *TLR4* up-regulation in partial SR (Study I). The relationship of sleep duration to inflammatory markers has been less clear in cohort studies (Solarz et al. 2012). Interestingly, I found *TLR4* and *IL1B* expression higher also in the epidemiological YFS sample among individuals with SSI (point-wise $P<0.05$ and <0.005, respectively; Fig. 2 in Study II). A study on TLR4-deficient mice found that the EEG measures of increased sleep drive

after sleep restriction were attenuated in these mice compared to wild-type mice, suggesting a role for TLR4 also in the generation of the sleep rebound *per se* (Wisor et al. 2011).

It is intriguing that although there are no foreign pathogens involved in sleep loss, it triggers activation of the immune system at least partly via the same signalling pathways as e.g. bacterial LPS (Figure 12). The known endogenous ligands of TLR4 are altered host-derived structures induced by injury or stress, such as heat-shock proteins (HSP) and products of extracellular matrix degradation (Keogh & Parker 2011). Seminal microarray studies on sleep loss in rodents have revealed up-regulation of immediate early genes and HSPs in the brain and liver (Cirelli et al. 2004, Maret et al. 2007). Whether HSPs are responsible for the TLR-mediated activation of inflammation by sleep loss remains to be further verified. In any case, sleep loss seems to pose a threat to the organism, triggering an immune response to protect the host against the threat. As a part of this response, many of the proinflammatory mediators induced by sleep loss, e.g. IL-1β and TNF-α, also increase sleep (Krueger et al. 2011). In case sleep loss is recurrent, it may lead to chronic low-grade inflammation, contributing to the development of atherosclerosis and various other diseases.

5.2.2 Transcription factors

The oPOSSUM analysis tool was used to identify transcription factor binding sites over-represented within the up- and down-regulated genes in the experimental SR study (Ho Sui et al. 2005). Binding sequences for transcription factors Prrx2, IRF2, Nobox, NFYA, STAT1, Pdx1, SRY, and Pax5 were enriched within 5.000 base pairs up- or downstream from the transcription site of the up-regulated genes ($P<0.05$; Study I). Of the genes coding for these transcription factors, the expression of interferon regulatory factor 2 (*IRF2*; pointwise P=0.020, and FC=1.50) and signal transducer and activator of transcription 1 (*STAT1*; pointwise $P = 0.027$, FC = 1.38) was up-regulated in SR.

IRF2 is a potential oncogene that has been suggested to participate in the regulation of NF-κB activity by modulating its subcellular localisation (Chae et al. 2008). STAT1 has been implicated to various functions, including TLR-signalling (Sikorski et al. 2012). These findings support the hypothesis that the inflammatory activation by sleep loss is at least partly mediated via TLR and NF-κB signalling.

The primary activator for STAT1 is the proinflammatory and proatherogenic cytokine interferon gamma (IFN-γ) (Sikorski et al. 2012). Contrary to the finding of induced STAT1 signalling in our SR experiment, we found that leukocytes extracted after SR released less IFN-γ upon stimulation (Study I). This would suggest an anti-atherogenic effect of sleep loss via decreased IFN-γ secretion. Thus, the complex regulation of the immune system activation in response to sleep loss needs further investigation.

Within the regions in the proximity of the down-regulated genes, binding sequences for transcription factors PBX1, Fos, RELA, GABPA, MYC-MAX, and Nobox were over-

represented ($P<0.05$). No down-regulation was observed in the genes coding for these transcription factors in SR compared to BL. Conversely, *Fos* expression was up-regulated in SR (pointwise $P=0.015$, FC=2.80).

5.3 Cholesterol transport pathways were down-regulated in sleep loss

The majority of the pathways enriched in the down-regulated gene set from the experimental SR study were related to lipid metabolism and transport (permuted $P<0.05$; Table S5 in Study I). The top-ranked (chole)sterol homeostasis and transport pathways were also enriched among the genes with lower expression in subjects with sleep loss in the epidemiological DILGOM sample (permuted $P<0.001$; Table 1 in Study II). The genes contributing to these cholesterol transport-related pathways were *ABCA1* and *NPC1* in the experimental SR study and *ABCG1*, *NPC1*, *NPCL1*, and *CAV1* in the DILGOM sample. The expression of these genes was assessed also in the YFS sample (N=1,407). *ABCG1* expression was lower in subjects with SSI also in the replication sample, supporting the suppressive effect of sleep loss on this cholesterol transporter.

ABCA1 codes a protein that belongs to the ABC family of transporters. ABCA1 is a ubiquitous protein expressed abundantly in liver, macrophages, brain, and various other tissues (Zannis et al. 2015). ABCA1 promotes efflux of cellular cholesterol and phospholipids to lipid-free or lipid-poor apoA-I, initiating the formation of HDL (Zannis et al. 2015). Inactivating *ABCA1* mutations, such as those found in Tangier disease, are associated to low cholesterol efflux, very low HDL cholesterol, and increased risk for atherosclerosis (Fitzgerald et al. 2010).

ABCG1 encodes another ABC transporter which promotes cholesterol efflux from cells to lipid-rich HDL, but not to lipid-free apoA-I (Zannis et al. 2015). Some studies in ABCG1-deficient mice have suggested a critical role for ABCG1 in promoting macrophage reverse cholesterol transport, while other studies in these mice have found no effects on plasma lipids, HDL, or other lipoproteins (Zannis et al. 2015). A genome-wide analysis study performed from monocytes collected from coronary artery disease (CAD) patients showed decreases in *ABCA1* and *ABCG1* expression compared to control subjects, although no difference in HDL levels was found (Sivapalaratnam et al. 2012). Our finding of decreased *ABCA1* and *ABCG1* expression could implicate a reduced potential of these monocytes to egress cholesterol to HDL acceptors (Oram & Vaughan 2006).

Niemann-Pick disease, type C1 (*NPC1*) was found, via a causative mutation explaining 95% of the cases of the Niemann-Pick disease. This lysosomal storage disorder is characterised by accumulation of cholesterol and sphingolipids, progressively leading to a fatal neurodegenerative course (Ikonen & Hölttä-Vuori 2004). Deficiencies of the intracellular cholesterol transporter NPC1 coded by the *NPC1* gene have been proposed to play a critical role also in atherosclerotic progression (Yu et al. 2014). NPC1 has been

shown to protect against atherosclerosis in mice by promoting the intracellular cholesterol trafficking in mice macrophages (Zhang et al. 2008). NPC1 transfers LDL-derived cholesterol from late endosomal/lysosomal vesicles to the endoplasmic reticulum for esterification or to plasma membrane for efflux. This is a crucial process governing the amount and distribution of intracellular cholesterol. The balance of cholesterol influx and efflux is important for maintaining proper cellular functions. If this process is impaired in the macrophages, they may develop into atherogenic foam cells. (Yu et al. 2014.)

The association of Niemann-Pick disease, type C1, gene-like 1 (*NPC1L1*) with atherosclerosis is contrary. *NPC1L1* inhibition has been suggested to have beneficial effects on atherosclerosis, metabolic syndrome, obesity, insulin resistance, and fatty liver. NPC1L1 facilitates the transfer of extracellular cholesterol to the cytoplasm of intestinal and liver cells. An NPC1L1-inhibiting drug, ezetimibe, has been shown to lower LDL cholesterol. (Betters & Yu 2010.) Heterozygous carriers of *NPC1L1*-inactivating mutations have also been shown to have lower LDL cholesterol and reduced risk for coronary heart disease (The Myocardial Infarction Genetics Consortium Investigators 2014). Thus, *NPC1L1* down-regulation in sleep loss might have a beneficial effect, contrary to the other cholesterol transporters down-regulated by sleep loss in our study.

Our results on lower expression of cholesterol transporter-coding genes *ABCA1*, *ABCG1*, and *NPC1* suggest that sleep loss may decrease RCT (Figure 13). Importantly, this was observed both in the experimental 5 nights' SR in controlled conditions and in real-life conditions in two independent epidemiological cohorts. The control of cholesterol levels is a complex system involving uptake, transport, biosynthesis, metabolism, and secretion. Interestingly, regarding the uptake, the expression of the CD36-coding gene was higher in experimental SR and in subjects with SSI in the epidemiological YFS sample ($P<0.05$ and $P<0.005$, respectively; Vilma Aho *et al.*, previously unpublished results). Together with the scavenger receptors, CD36 is centrally responsible for the uptake of cholesterol by macrophages (Hansson & Libby 2006). Thus, this finding could suggest an increased cholesterol uptake into macrophages, possibly leading to the development of foam cells, central players in the pathophysiology of atherosclerosis. The complexity of the regulation of cholesterol metabolism and transport calls for further studies to deepen the understanding on the effects of sleep loss on cholesterol and cholesterol-related diseases.

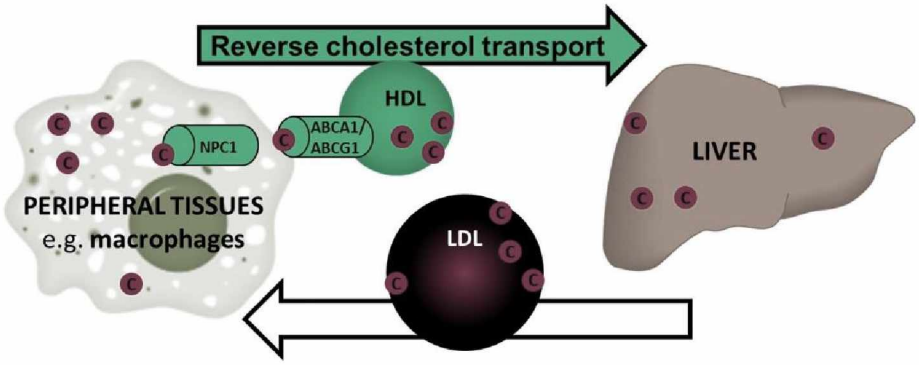

Figure 13. Sleep loss may decrease reverse cholesterol transport
A simplified cartoon illustration depicting high density lipoproteins (HDL, respectively) and cholesterol transporters in reverse cholesterol transport. Low density lipoproteins (LDL) transport cholesterol (and other lipids) to tissues for uses e.g. in membranes and steroid hormone synthesis. Excess cholesterol is transported back to the liver by HDL. The cholesterol transporters coded by genes ABCA1, ABCG1, and NPC1 – down-regulated in sleep loss in our study – participate in the reverse cholesterol transport by transporting cholesterol from the tissues to the HDL. In case of impaired reverse cholesterol transport, cholesterol may accumulate in the tissues (e.g. macrophages), possibly leading to plaque-formation in the arteries. This process forms part of the development of atherosclerosis, and may in the long run result in a stroke or heart attack.

Sleep loss seems to affect points of convergence between various functions. These hubs of regulation are in the crossroads of the regulation of physiological systems. One point of integration of the immune responses and lipid metabolism is the regulation via liver X receptors (LXR). LXR activation may inhibit atherosclerosis inhibition and development by promoting the transcription of cholesterol efflux-related genes and inhibiting the expression of several proinflammatory transcripts (Joseph et al. 2003, Lee & Tontonoz 2015). The expression of *ABCG1* can be induced by LXR agonists in the liver and macrophages, and by cholesterol loading in macrophages (Zannis et al. 2015). LXR agonist-treatment has been reported to increase *ABCA1* and *NPC1* expression, HDL cholesterol and total cholesterol, and macrophage cholesterol-efflux, and reduce aortic atherosclerotic lesions in apolipoprotein E-deficient mice (Dai et al. 2008). The findings of decreased expression of cholesterol transport-related genes *ABCA1*, *ABCG1*, *NPC1*, and activation of proinflammatory mediators, such as IL-1β, *MYD88*, and TLR-coding genes, led us to hypothesize that LXR could be involved in the effects of sleep loss (Study II). However, a decrease in LXR was not observed directly in our data, and the hypothesis of decreased LXR activation as a mediator of the proinflammatory activation and decreased RCT in sleep loss could be a starting point for future studies.

5.3.1 Methodological issues in transcriptomics

The reliability of the detection of gene expression using microarrays has been questioned, and validation with another method is often requested (Rajeevan et al. 2001). To validate the results in our SR study, the expression changes of six genes were assessed also with

qPCR. The microarray-detected up- or down-regulation of five of the six genes was confirmed by qPCR ($P<0.05$; Study I). This technical replication supports the validity of our gene expression detection by microarrays.

The transcriptomics was assessed in peripheral blood samples, and not e.g. liver or brain tissue. Although circulating biosignatures from leukocytes or whole blood have been suggested as an "accessible window to the multiorgan transcriptome" and useful tools for detecting biomarkers of various diseases in other tissues (Moore et al. 2005, Kohane & Valtchinov 2012), our results mainly describe the effects of sleep loss in the white blood cells. As many of the atherosclerotic processes occur in leukocytes, blood is one relevant tissue to study the effects of sleep loss in the development of atherosclerosis. However, as the liver plays a major role in the metabolism of cholesterol and other lipids important in atherosclerosis, further studies focusing on the hepatic processes in animal models are needed to complete the picture. One transcriptomics study focusing on expression changes in the mouse brain used liver as a reference tissue (Maret et al. 2007). Surprisingly, sleep loss induced nearly three times more expression changes in the liver than in the whole brain, suggesting a major effect of sleep loss on liver function at the transcriptional level.

Gene expression overall tells only a part of the story, as many physiological processes are regulated at several levels, not only at the level of transcription. E.g. translation from RNA to protein, post-translational modifications, activation of receptors by binding of ligands, and protein degradation are involved in the regulation of various processes. However, transcriptomics is a considerable means for finding affected pathways and creating new hypotheses. The discovered pathways have to be further examined on a molecular level to elucidate mechanisms involved.

5.4 Lipoprotein profiles were altered by sleep loss

5.4.1 NMR metabolomics

NMR metabolomics revealed that the lipoprotein profiles were altered in sleep loss (Figure 14) (Study II). Epidemiological SSI in the DILGOM sample was associated to lower large HDL ($P<0.05$). This finding was confirmed in the YFS replication sample ($P<0.05$).

Surprisingly, the concentration of small, medium, and large LDL and small VLDL decreased in our SR experiment compared to BL ($P<0.01$). The LDL-associated apolipoprotein apoB also decreased in SR ($P<0.005$). Large and extra-large HDL showed a trend for an increase in SR (point-wise $P<0.05$, not significant after correction for multiple testing), and no change was observed in medium or small HDL.

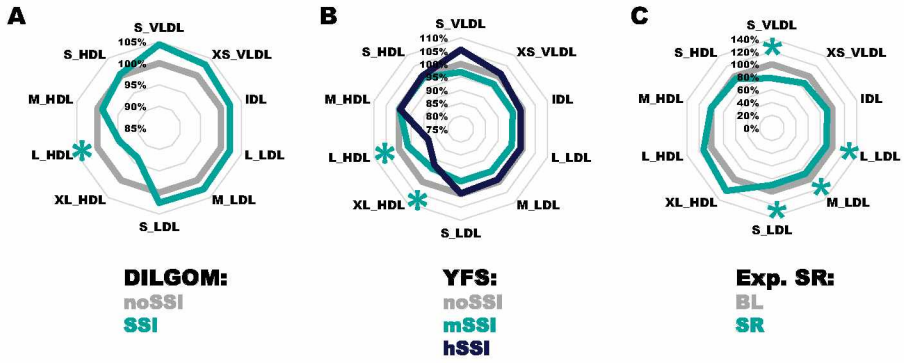

Figure 14. Lipoprotein particles in sleep loss
Serum very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) divided into subtypes based on size using NMR metabolomics. (Large VLDL and chylomicron classes are not shown as their concentrations in fasting blood samples are extremely low.) **A)** DILGOM subjects (N=340) with SSI compared to those with noSSI (mean concentration of each lipoprotein class in noSSI subjects shown as 100%), **B)** Young Finns Study replication sample (N=2,077) subjects with heavy (hSSI) or moderate SSI (mSSI) compared to noSSI, and **C)** experimental sleep restriction (SR) compared to baseline (BL) (N=14). Modified from Study II.

Few studies have investigated the association of sleep duration with cholesterol measures. The findings have been inconsistent. In line with our results on lower HDL in subjects with insufficient sleep, we have earlier reported slightly lower HDL cholesterol with short sleep duration (Ollila et al. 2012). Also a study in Norwegians found lower HDL cholesterol concentrations associated with shorter sleep durations (Bjorvatn et al. 2007). Also a recent Japanese prospective study reported a higher hazard ratio for low HDL cholesterol in short (5–<7 h) sleepers during a 6-year observation period (Kinuhata et al. 2014). Another Japanese population study found an increased risk only in women (Kaneita et al. 2008). Some studies have suggested short sleep as a risk factor for hypercholesterolemia in both sexes (Bjorvatn et al. 2007, Choi et al. 2008, Gangwisch et al. 2010). On the contrary, one study has reported higher total cholesterol and total/HDL cholesterol ratio with longer sleep durations in adult population (Petrov et al. 2013) and one in the elderly (van den Berg et al. 2008).

Experimental sleep restriction (4 h nightly sleep for 3 nights) has been earlier reported to increase total and LDL cholesterol in postmenopausal women treated with hormone replacement therapy (Kerkhofs et al. 2007). Interestingly, in our SR study, LDL decreased (Study II). The very different study populations may explain these contradictory results. The LDL decrease – a beneficial effect regarding the traditional cardiovascular risk evaluation – may be connected to the immune response triggered by sleep loss. Immune system activation has been shown to induce modifications in lipid

metabolism (Khovidhunkit et al. 2004). I suggest that acute phase response could explain decreases in cholesterol in response to sleep loss.

NMR metabolomics also yields information on various lipids (such as fatty acids, triglycerides, and phospholipids), amino acids (8 of the 21 eukaryotic amino acids can be detected), and small molecule weight energy metabolites (such as glucose, lactate, citrate, and 3-hydroxybutyrate) (Table S3 in Study II) (Soininen et al. 2009). While no significant differences were observed in the other lipid species, amino acids or other metabolites, the concentration of omega-6 (ω-6) fatty acids decreased in experimental SR ($P<0.005$; Table S3 in Study II). The literature on the impact of this group of polyunsaturated fatty acids on cardiovascular disease risk has been somewhat controversial. Some studies have showed a protective association while others have suggested harmful effects via proinflammatory activation (Wu et al. 2014). A recent systematic review on randomised-controlled trials found no effects of ω-6 supplements in the prevention of cardiovascular diseases (Al-Khudairy et al. 2015). Thus, it remains to be clarified whether the ω-6 fatty acid linoleic acid (18:2) decrease in SR ($P<0.0005$) might also contribute to the higher risk for developing atherosclerosis.

To my knowledge, NMR-based metabolomics has not been earlier used for studying blood metabolites in experimental nor epidemiological sleep loss. Recently, one study assessed urine NMR-metabolomes after short-term sleep deprivation (Weljie et al. 2015). This study reported oxalic acid and diacylglycerol 36:3 as cross-species markers for sleep loss.

5.4.2 Lipid transfer protein and enzyme activity

LCAT is crucial in the initial formation of HDL, by interacting with lipid-bound apoA-I to esterify free cholesterol in this pre-HDL particle, forming spherical HDL particles (Zannis et al. 2015). CETP and PLTP are major transporters of lipid species – namely cholesterol, triglycerides and phospholipids – between the lipoprotein particles (Zannis et al. 2015). To investigate whether these enzymes and lipid transporters accounted for the sleep loss-induced changes in lipoproteins, their activities were measured in serum samples from the experimental SR study. No changes were found in SR compared to BL (Study II), suggesting that other mechanisms account for the modifications observed in the lipoprotein pool.

APR has been proposed to change HDL from anti-inflammatory to proinflammatory (Van Lenten et al. 1995). PON1 is an atheroprotective enzyme linked to anti-inflammatory and antioxidative effects of HDL (Karlsson et al. 2015). We hypothesised that sleep loss could change the anti-inflammatory properties of HDL via APR. However, no changes were observed in the activity of PON1 (Study II).

5.5 Rest homeostasis was confirmed in zebrafish

To confirm sleep homeostasis in zebrafish larvae, I developed a system for rest-depriving this model organism with a naturalistic stimulus and test their responses to sensory

stimuli (Study III). Startle responses after rest deprivation were quantified using high-speed video recording and automatic behavioural detection software.

Rest deprivation by constant flow of water decreased the sensory responsiveness of zebrafish larvae (Figure 15). Rest-deprived larvae showed a startle response to an electrical stimulus less likely than control larvae ($P<0.05$). No differences were found in the amount of short latency C-starts (SLC), while the long latency C-starts (LLC) decreased. This fits the hypothesis of sleep as a function of networks of the brain, as SLC is considered a reflex-like behaviour with a short circuitry, while LLC involves more central processing (Kimmel et al. 1980, Gahtan et al. 2002, Korn & Faber 2005, Burgess & Granato 2007, Tabor et al. 2014).

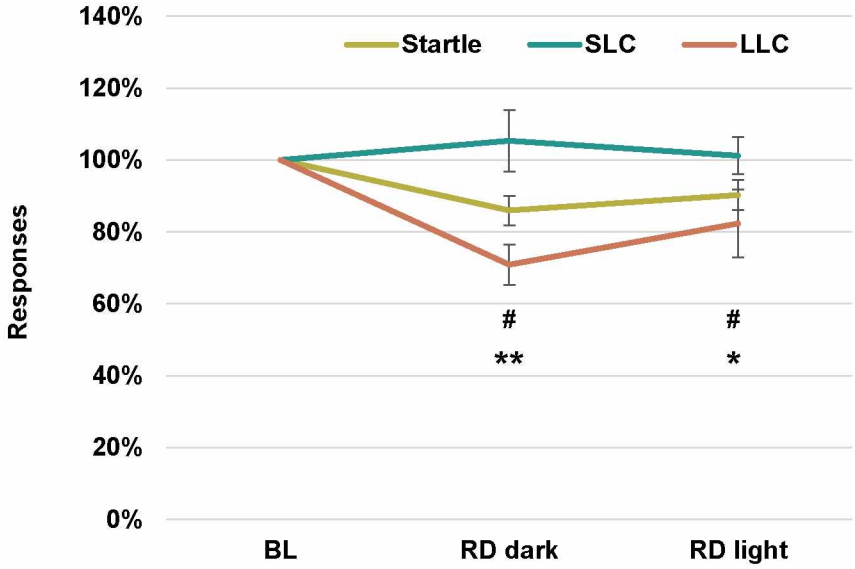

Figure 15. Rest deprivation decreased sensory responsiveness
After 6 h of night-time water flow, 1-week old zebrafish larvae responded less to electrical stimuli. Compared to baseline (BL), startle responses and especially long latency C-starts (LLC) decreased, while no changes were observed in short latency C-starts (SLC). # = P(startle)<0.05; * = P(LLC)<0.05; ** = P(LLC)<0.005. Data represent BL- and control-normalised means±s.e.m. Modified from Study III.

In previous studies, rest/sleep rebound has been shown only if the zebrafish were kept in the darkness after the sleep deprivation (Zhdanova et al. 2001, Prober et al. 2006, Yokogawa et al. 2007). This has been interpreted as a sign of a non-existent or weaker sleep homeostasis that can be overwritten by the activating effect of light (Rial et al. 2007, Sigurgeirsson et al. 2013). However, in our study, the rest-deprived larvae showed less LLCs during both the remaining dark phase ($P<0.0005$) and the first hours after lights were switched on at the regular time (9:00, ZT0; $P<0.05$). A similar flow protocol during

daytime did not decrease responsiveness to sensory stimulation. I suggest that the observed decreased sensory responsiveness after night-time rest deprivation is a verification of sleep homeostasis in this model.

I hypothesised that the water flow is a more naturalistic method for rest deprivation than e.g. electrical shocks. Stress levels were not measured in this study, but this could be tested in further experiments.

Habituation to the rest deprivation protocol was not observed across the night. According to our analysis of the orientation of the larvae during the flow protocol, the majority of the larvae in the rest deprivation group were swimming against the flow during the whole 6 h rest deprivation, while the control larvae were oriented randomly to all directions (Figure 16).

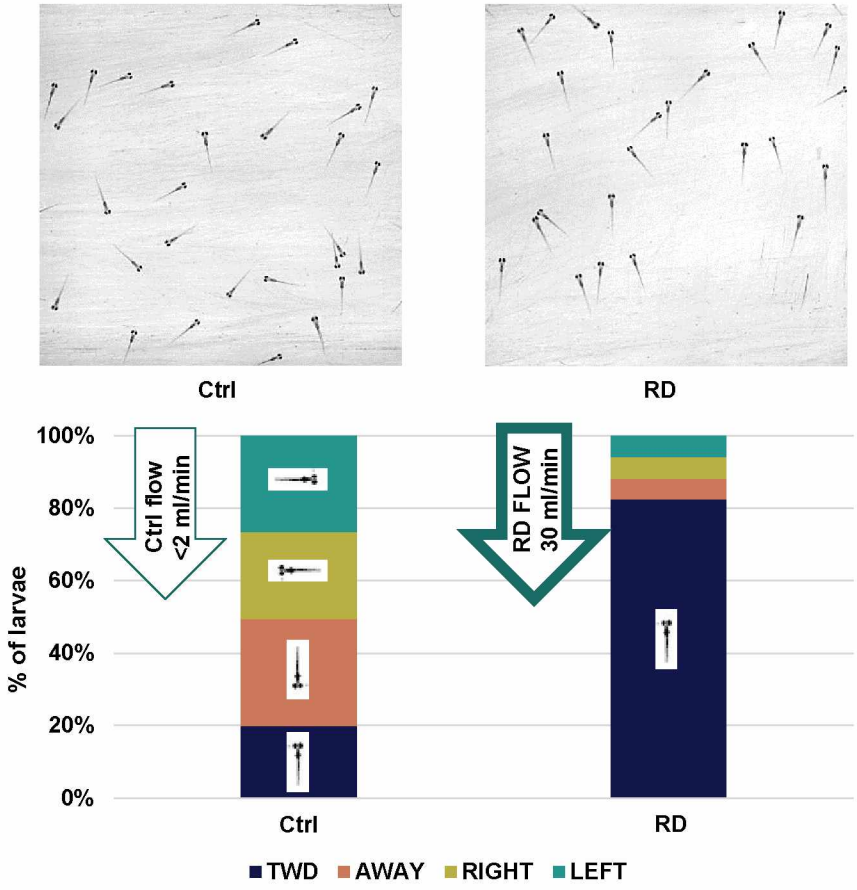

Figure 16. Rest deprivation by swimming against water flow
Over 80% of the larvae being rest-deprived (RD) were oriented towards (TWD) the flow during the night, while in the control (Ctrl) group, larvae were randomly oriented. Modified from Study III.

High speed recording (1000 images/s) provides an appropriate time resolution to detect the fast escape responses that occur in the time scale ranging from milliseconds to tens of milliseconds in larval zebrafish (Burgess & Granato 2007). I propose that these methodological advancements enabled the detection of rest rebound in larval zebrafish, also in lights on conditions

5.5.1 Adenosine

In addition to behavioural experiments, pharmacological experiments were used to study sleep homeostasis in zebrafish larvae. Adenosine has been shown to increase during sleep deprivation the basal forebrain of rodents, promoting NREM sleep rebound (Porkka-Heiskanen et al. 1997). Adenosine receptor agonists increase sleep and activate the VLPO in many species, while antagonists, such as caffeine, decrease sleep (Porkka-Heiskanen & Kalinchuk 2011). Polymorphisms of adenosine metabolism or signalling-related genes have been suggested to contribute in NREM sleep homeostasis in humans (Landolt 2008).

In our study, adenosine receptor A1 agonist cyclohexyladenosine administered to swimming water as 100 µmol/l during daytime decreased startle responses elicited by electrical stimuli ($P<0.05$; Study III). CHA also increased immobility bouts longer than 60 s, defined as sleep, when given at daytime ($P<0.05$; Study III).

Caffeine, an adenosine A1 and A2a receptor antagonist (100 µmol/l), decreased >60 s long immobility bouts when given at night-time ($P<0.001$; Study III). The decrease was not statistically significant if caffeine was administered at daytime.

These findings are in line with the few earlier studies on adenosine and sleep in zebrafish. A screen of nearly 4000 small molecules showed that adenosine agonists increase immobility bouts defined as sleep, while antagonists decrease these bouts (Rihel et al. 2010a). Another study found that the sleep-promoting effects of melatonin are partly mediated by adenosinergic signalling (Gandhi et al. 2015).

The involvement of adenosinergic modulation in the sleep/wake states supports the hypothesis that sleep homeostasis exists in zebrafish larvae and is conserved at the molecular level.

5.5.2 Methodological considerations for zebrafish studies

When designing the method for rest deprivation and escape response detection, several aspects of the natural habitat and behaviour of zebrafish larvae were taken into consideration. The experimental conditions were adjusted to resemble the conditions found in nature and widely accepted for laboratory experiments for this species. Temperature was kept close to +28.5°C, pH neutral, and light–dark cycle constant at 14h/10h. Most importantly, a natural stimulus, water flow, was chosen for the rest deprivation protocol. Although light is a strong natural arousal-promoting stimulus for

zebrafish, it was not used for the rest deprivation (as has been done in some other studies) to avoid effects for the circadian timing.

Yet, even though the flow of water is a natural stimulus in the natural habitat of zebrafish larvae, swimming against the flow may cause physical exhaustion from the muscular "endurance exercise". Even though the effect on startle responses was controlled by applying a similar flow protocol to larvae during the day and no effect was found (Study III), it is important to bear in mind that the exercise increases energy expenditure compared to control larvae and may affect some aspects of physiology. This needs to be carefully controlled in future studies utilising this rest-deprivation method, especially in those targeting changes in metabolism.

Electrical stimuli were selected to elicit startle responses because they can be given precisely on certain time point, their strength can be easily modulated, and they do not continue after the given duration or move the medium (like acoustic vibrations may). However, the stimulation may cause some stress on the larvae, and this should be assessed in future studies.

Also, only one voltage was used to test the responses. To further develop the method, arousal threshold could be tested with several stimuli of increasing intensities, e.g. starting from 1 V and increasing voltage up to 7 V, to yield more precise information on the arousal threshold of each larva.

In the adenosinergic experiments, only one concentration of adenosine agonist or antagonist was studied. In future studies, several concentrations could be used to test for possible dose-dependent effects.

6 Conclusions

The effects of sleep loss were studied in experimental and real-life conditions in humans (studies I and II). The laboratory experiment was a simulation of a working week with sleep restricted to 4 hours per night. Two epidemiological samples from Finnish population were used to estimate the effects of self-reported sleep insufficiency. In both the experimental and epidemiological samples, effects of sleep loss on transcriptomic and metabolomics profiles were assessed. To the best of my knowledge, this was the first study to screen sleep-related changes in blood metabolites using NMR metabolomics. Also, sleep-related transcriptomes had not been previously reported from population cohorts.

Our results show that sleep loss activates the immune system and down-regulates reverse cholesterol transport at the level of gene expression (studies I and II). If sleep loss becomes chronic, it may decrease large HDL in the blood. These changes may participate in the complex development of atherosclerosis, and partly explain the increased risk for cardiometabolic diseases (Figure 17), earlier reported in individuals with short or insufficient sleep. This adds to the burden of other atherogenic physiological and lifestyle factors, such as hypertension, insulin resistance, unhealthy diet, and sedentary behaviour, which are also affected by sleep loss.

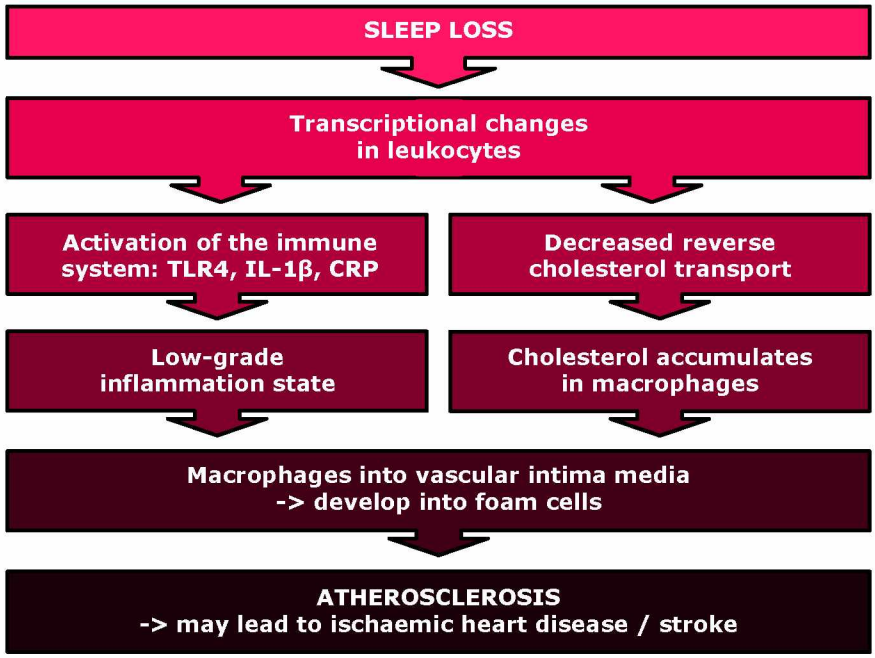

Figure 17. Sleep loss may increase atherogenic processes by activating inflammation and decreasing reverse cholesterol transport (RCT)
A hypothesised model based on the observations at the level of transcription and serum lipoproteins in this body of work (studies I and II).

In addition to the human studies, a method was developed for sleep research in zebrafish larvae (Study III). I propose that water flow is a naturalistic method for rest-depriving larval zebrafish, resembling the gentle handling protocol developed for rodent experiments with the aim of decreasing stress in sleep deprivation. The larvae showed a rest rebound after rest deprivation applied during the night with this method. This sign of rest homeostasis was observed also after lights were switched on, which earlier studies in zebrafish had failed to show. Zebrafish larvae can now be used in further studies of the effects of sleep loss on immune functions and metabolism.

The current findings add to the pondering of Aristotle and his posterity; sleep is for the brain *and* the body, and it appertains to at least the immune system and metabolism in addition to neural functions. All animals thus far studied, including fish, seem to partake in both sleep and waking. Nevertheless, the ultimate question of the field still remains: *from what cause it arises that sleep and waking are attributes of animals* i.e. why do we sleep?

7 Future Directions

As research tends to do, these studies have provided more information on some specific questions while raising a fair amount of new questions.

We observed signs of decreased reverse cholesterol transport at the level of cholesterol-transporter gene expression in leukocytes in both epidemiological and experimental sleep loss, and lower large HDL in epidemiological sleep loss (Study II). To confirm the effects of sleep loss on RCT, the efflux of cholesterol from macrophages to HDL could be studied *in vitro* in blood samples drawn from sleep-restricted and control subjects. Also, zebrafish may be a valuable model for imaging studies elucidating the effects of sleep loss on cholesterol metabolism and transport. It has been successfully used in research on lipid metabolism and atherosclerosis, and with the rest deprivation method described in Study III, it can be utilised also to study the effects of restricted sleep.

Sleep loss has been consistently shown to activate the immune system and inflammatory mediators, but it is not clear what, at molecular level, is the initial activator. What triggers the immune response in the first place, as there are no foreign pathogens, such as bacteria or viruses, involved? The toll-like receptors were up-regulated at the transcriptional level experimental and epidemiological sleep loss in our study. In addition to the pathogen-associated molecule patterns, TLRs have also endogenous ligands. These are markers of cellular damage that also activate proinflammatory signalling via TLRs. Could this be the point where sleep loss initiates the activation of the immune system? And if so, what type of cell damage sleep loss originally causes? Genetically modifiable animal models, including zebrafish, may offer novel information on the mechanisms of the immune response activation in sleep loss.

The time course of the events could also be studied further. Experimental sleep restriction could help to elucidate how the LDL decrease switches into lower HDL? Is it the inflammatory activation that changes the regulation of cholesterol metabolism, or *vice versa*, or does sleep loss affect both systems independently? How short sleep curtailment is enough to alter the immune response and cholesterol metabolism? How long time is needed for the various systems to recover to baseline levels, and what kind of carryover effects can occur if the sleep curtailment is repeated? Prospective epidemiological cohorts could give causative information on the long-term effects – whether real-life sleep loss really has predictive value on e.g. atherogenic changes in cholesterol transport. Zebrafish larvae may be used to study the time course of the effects of sleep loss in highly controlled and reproducible conditions.

Also sex differences and inter-individual variation are of interest in further studies. Many epidemiological studies have reported sex differences in the association of sleep phenotypes and the risk for cardiovascular diseases. Lipid-related measures have substantial differences between genders, which could be further investigated regarding sleep.

Large variation was also found between subjects in various physiological measures, as well as cognitive performance. Some of the variability was present already at the baseline, but also the tolerance of sleep loss varied. Are there genetic differences that could partly explain why some individuals are more susceptible to sleep loss, while others survive with very little sleep? How to address individual's sleep (e.g. sleep need, duration, sufficiency, quality etc.) more objectively at the population level to better bridge the gap between the experimental and epidemiological studies?

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